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Edited by Shu-You Peng, MD, FACS(Hon), Professor, Min Wang, MD, PhD, and Ren-Yi Qin, MD, FACS, Professor

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W C P World Clinical Pancreatology

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EDITORS FOR THIS BOOK

Responsible Electronic Editor: *Su-Qing Liu*
Proofing Editorial Office Director: *Jin-Lei Wang*

8226 Regency Drive, Pleasanton, CA 94588-3144, United States
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
http: //www.wjgnet.com

PREFACE

Core progress of clinical pancreatology in 2014

It is with great pleasure that we introduce the first edition of the *World Clinical Pancreatology* (ISBN 978-0-9914430-4-8), published by the Baishideng Publishing Group in 2015; this compendium of review articles highlights the up-to-date research in clinical pancreatology as well as the ongoing controversial issues in this field. The 123 included articles, contributed by authors from 24 countries who are recognized for their excellent work in the field, provide a comprehensive introduction to the current clinical management strategies in pancreatology. These articles also provide insights into the most recently developed and proposed strategies, including genetic detection techniques, early detection biomarkers, and improvements upon traditional imaging methods for diagnosis. The presentation of controversial perspectives related to treatments of neoadjuvant strategies, surgery, chemotherapy and immunization will be of particular interest to readers involved in the field of clinical management of pancreatic conditions and diseases. Finally, the inclusion of basic research studies promotes the practice of translational research, providing data that will stimulate the development and application of new clinical treatment strategies to improve pancreatic health.

The articles in this book were selected from the following journals: *World Journal of Gastroenterology*, *World Journal of Hepatology*, *World Journal of Gastrointestinal Endoscopy*, *World Journal of Gastrointestinal Oncology*, *World Journal of Gastrointestinal Pathophysiology*, and *World Journal of Gastrointestinal Surgery*. The compendium is organized according to subspecialty, with one article focusing on exocrine pancreatic insufficiency, one on pancreatic cyst, 78 on pancreatic neoplasms, and 43 on pancreatitis. We appreciate all of the authors' outstanding work assembled herein. Although this book is directed at scientists and physicians working in clinical pancreatology, the concepts and strategies reported in the collected articles are also expected to serve as a source of inspiration to all medical practitioners and researchers with interests in the field. This feature should stimulate productive discussions among the scientific and medical communities and facilitate translation of research data to clinical practice, ultimately promoting our collective efforts towards reaching a consensus for standard treatment strategies in pancreatology and majorly benefiting patients as well as their caregivers.

Shu-You Peng, MD, FACS(Hon), Professor

Min Wang, MD, PhD

Ren-Yi Qin, MD, FACS, Professor

Shu-You Peng, Department of Surgery, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310009, Zhejiang Province, China
Min Wang, Ren-Yi Qin, Department of Pancreatic-Biliary Surgery, Affiliated Tongji Hospital, Tongji Medical College, Huazhong Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

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Pancreatic enzyme replacement therapy for pancreatic exocrine insufficiency in the 21st century

Tony Trang, Johanna Chan, David Y Graham

Tony Trang, Johanna Chan, David Y Graham, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, Houston, TX 77030, United States

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Correspondence to: David Y Graham, MD, Professor, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, 2002 Holcombe Blvd, Houston, TX 77030, United States. dgraham@bcm.edu

Telephone: +1-713-7950232 **Fax:** +1-713-7901040

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Abstract

Restitution of normal fat absorption in exocrine pancreatic insufficiency remains an elusive goal. Although many patients achieve satisfactory clinical results with enzyme therapy, few experience normalization of fat absorption, and many, if not most, will require individualized therapy. Increasing the quantity of lipase administered rarely eliminates steatorrhea but increases the cost of therapy. Enteric coated enzyme microbead formulations tend to separate from nutrients in the stomach precluding coordinated emptying of enzymes and nutrients. Unprotected enzymes mix well and empty with nutrients but are inactivated at pH 4 or below. We describe approaches for improving the results of enzyme therapy including changing to, or adding, a different product, adding non-enteric coated enzymes, (*e.g.*, giving unprotected enzymes at the start of the meal and acid-protected formulations later), use of antisecretory drugs and/or antacids, and changing the timing of enzyme administration. Because considerable lipid is emptied in the first postprandial hour, it is prudent to start therapy with enteric coated microbead prior to the meal so that some enzymes are available during that first hour. Patients with hyperacidity may benefit from adjuvant antisecretory therapy to reduce the duodenal acid load and possibly also sodium bicarbonate to prevent duodenal acidity. Comparative studies of clinical effectiveness of different formulations as well as the characteristics of dispersion, emptying, and dissolution of enteric-coated microspheres of different diameter and density are needed; many such studies have been completed but not yet made public. We discuss the history of pancreatic enzyme therapy and describe current use of modern preparations, approaches to overcoming unsatisfactory clinical responses, as well as studies needed to be able to provide reliably effective therapy.

Key words: Pancreatic insufficiency; Pancreatic enzyme replacement therapy; Lipase; Clinical trials; Steatorrhea; Fat malabsorption; Chronic pancreatitis

Core tip: In the last two decades, a number of studies comparing pancreatic enzymes and placebo have confirmed that pancreatic enzymes are superior to placebo for treatment of pancreatic malabsorption. While many patients achieved a satisfactory clinical response, individualization is often needed. Studies conclusively show that dose escalation is not a reliable method of obtaining further improvements and instead results in increased costs. Here, we describe alternate strategies for obtaining a satisfactory clinical response including changing to, or adding, a different product, adding non-enteric coated enzymes, use of antisecretory drugs and/or antacids, and changing the timing of enzyme administration.

Figure 1 David Y Graham, MD, Professor, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, 2002 Holcombe Blvd, Houston, TX 77030, United States.

BIOGRAPHY

David Y Graham (Figure 1), MD is a Professor in the Departments of Medicine and Molecular Virology and Microbiology at Baylor College of Medicine, in Houston, TX. He received his undergraduate degree from the University of Notre Dame in South Bend, Indiana, his MD degree with honor from Baylor University College of Medicine in 1966. He board certified in Medicine and Gastroenterology. Dr. Graham is a Past President of the American College of Gastroenterology. He is the Editor of the journal *Helicobacter*. His primary interests are related to infections of the gastrointestinal tract tract including *Helicobacter pylori*, Norovirus infections, and the infectious etiology of inflammatory bowel disease.

Dr. Graham is internationally recognized for his expertise in medicine and gastroenterology and is the author of more than 900 scientific papers, several books, and more than 100 chapters in medical text books. One of his papers is listed as one of the three most important papers in gastroenterology in the first 80 years of the *Annals of Internal Medicine*: (*i.e.*, Landmark Papers in Internal Medicine: The First 80 Years of Annals of Internal Medicine. Harold C Sox and Edward J Huth (Eds), 2009 (paper cited: Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann Intern Med* 1992; 116: 705-8.).

He is a Master of the American College of Gastroenterology and a Fellow of the American College of Physicians, the American Academy of Microbiology, the American Association for the Advancement of Science, the Infectious Diseases Society of America, and World Innovation Foundation. He is a past president of the American College of Gastroenterology and the winner of many prestigious awards. He previously was a physician to NASA astronauts during the Apollo program. He is listed as among the Top 50 Most Influential Gastroenterology Professionals of the 20th Century by Gastroenterology.com, as one of ISI's Highly Cited Researcher in Clinical Medicine, and as one of the Best Doctors in America. He has patents regarding development of diagnostic tests for *Helicobacter pylori* infection, the cause of peptic ulcer and gastric cancer and for vaccine development of Norwalk virus infection, the most common cause of food borne and cruse ship associated diarrhea.

INTRODUCTION

Orally administered pancreatic enzymes have been avail-able since at least the 19th century, when many formulations were available as digestive aids. At that time it was known that orally administered enzymes were destroyed in gastric juice and that they were most effective when given in alkaline media[1]. A review of early 20th century research on the use of pancreatic enzymes for treatment of steatorrhea secondary to exocrine pancreatic insufficiency reported a wide variation in efficacy, yielding an overall 50% approximate reduction in steatorrhea[2]. The goal of pancreatic enzyme therapy is to restore normal fat absorption by delivering "a sufficient amount of active lipase at the right place, *i.e.*, duodenum and proximal jejunum, and at the right time, *i.e.*, in parallel with gastric emptying of nutrients"[3]. Achieving this goal has remained elusive despite the introduction and use of modern potent enzyme preparations[3-9].

Normal fat absorption requires integration of nutrient delivery with pancreatic and biliary secretions to accomplish hydrolysis and solubilization of ingested fats and fat-soluble dietary constituents. The normal process is finely tuned and requires coordination of many steps including controlled delivery of nutrients to the intestine, neutralization of acidic gastric contents, and secretion of pancreatic enzymes and bile to promote optimal digestion and solubilization of digestive products. These products of digestion then require a sufficient luminal intestinal surface area for absorption. Normally, the intestinal tract is able to process and absorb approximately 95% of ingested fat. There is considerable reserve capacity with all of the elements such that major anatomic alterations are required for weight loss surgery to be effective. The pancreas provides the bulk of the lipase needed for hydrolysis of triglycerides as well as bicarbonate to neutralize the acidic gastric contents. Pancreatic steatorrhea generally does not occur until lipase secretion is reduced by 90% or more[10].

Pancreatic steatorrhea is caused by disruptions of the normal process in which pancreatic enzymes are either inactivated or are otherwise unavailable (*e.g.*, blockage of the pancreatic duct, or resection or destruction of the glandular pancreas). Fungal, plant, and animal (especially porcine) pancreatic enzymes are available, and theoretically the simple addition of these enzymes with meals should resolve the deficiency and restore normal absorp-

Table 1	Reasons for a poor response to supplemental enzyme therapy

Inactivation of the enzymes in the stomach by acid and/or proteases
Inadequate mixing of the enzymes and nutrients during delivery to the small intestine such that a proportion of the meal is not exposed to appropriate concentrations of enzymes
Separation of enteric-coated microspheres from meal contents in the stomach
Low duodenal and small bowel pH fail to provide optimal conditions for lipase and bile salts to provide optimal digestion of the ingested nutrients
Delayed dissolution of enteric-coated enzyme microspheres in the small intestine
Incorrect or incomplete diagnosis

Table 2	Myths regarding modern microbead enzyme therapy

Currently available formulations will reliably correct steatorrhea
Increasing the dose of microbeads increases the effectiveness
Choice of dose depends on fat content of the diet
Proton pump therapy generally improves success with microbead therapy
Microbeads are fully protected in applesauce
Uncoated enzymes have no place in modern pancreatic enzyme therapy

tion. Despite this hypothetical possibility, the administration of large doses of replacement pancreatic enzymes generally has not resulted in complete restoration of normal fat absorption[2,9,11-14].

One early approach was the use of enteric coating to protect the enzymes during passage through the stomach, but this was met with limited success[2,15]. Subsequent studies of normal gastric and pancreatic physiology identified many other barriers to successful treatment with pancreatic enzymes[16,17] (Table 1). This paper discusses the current status and clinical effectiveness of pancreatic enzyme therapy as well as possible approaches to overcoming the barriers to successful therapy. We also discuss the many myths and common misconceptions regarding therapy (Table 2). We begin with a historical review of the use of pancreatic enzyme therapy in the treatment of malabsorption due to chronic pancreatitis and cystic fibrosis; this historical perspective also provides the physiologic basis for the use of supplemental pancreatic enzymes and adjuvant therapies. We focus on overcoming the limitations of common strategies used to improve outcome, such as increasing the amount of lipase per meal, use of enteric-coating, the timing of enzyme administration in relation to meals, and use of antacids and antisecretory drug as adjuvant therapy. Success requires a strategy that is targeted to identify and overcome the specific barriers preventing correction of steatorrhea (Table 1). Currently, many patients achieve a satisfactory clinical response but few experience complete normalization of fat absorption; more than half often require individualized therapy to obtain symptomatic and nutritional relief[3-8].

The review is based on understanding the underlying physiology and the results of clinical trials in patients. It does not seek to comprehensively review all studies but rather to illustrate key principles and to show consistency of the results (typically failures to achieve correction of steatorrhea). Although meta-analyses have confirmed that enzyme therapy is superior to placebo, there is no evidence that one product is superior to another or that any will reliably eliminate steatorrhea. We also do not consider potential alternate indications for pancreatic enzymes such as abdominal pain in patients with chronic pancreatitis[18] or irritable bowel syndrome[19,20].

MODERN ERA OF PANCREATIC ENZYME THERAPY

In 2004 the United States Food and Drug Administration (FDA) issued a requirement for manufacturers of prescription pancreatic enzyme products to submit new drug applications (NDAs) for all pancreatic enzyme products[21]. The FDA provided guidance on the minimal standards regarding the amount and stability of enzymes and the studies needed to establish efficacy (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071651.pdf). The companies were told that only products receiving a new FDA approval would be allowed to remain on the market by 2008; this was later extended to 2010. The primary efficacy requirement was based on the comparison of the active product with placebo, which set a relatively low bar for efficacy. The FDA also requested, but did not require for approval, additional information about each product in terms of studies addressing gastric emptying, mixing, and dissolution time. The majority of products now available in the United States are enteric coated and formulated as microbeads, microtablets or microspheres (we use the terms "microbeads", "microtablets" and "microspheres" interchangeably). A non-enteric-coated product (Viokaze®, Forest Pharmaceuticals) was approved in 2012 (Table 3).

Most of the formulations are marketed in different strengths based on enzyme activity per capsule or tablet. Increasing the activity/dosage unit has generally been achieved by re-packaging the basic enzyme product into larger capsules, using different diameter enteric-coated beads, or both (Figure 2, Table 3).

The available prescription products are relatively expensive (Table 3). However, because "health food" stores still offer pancreatic enzymes as non-prescription "digestive aids" at a relatively low cost, many patients are likely to also use them. As noted, none of the currently available approved formulations have been shown to reliably achieve normal absorption irrespective of the quantity of lipase administered.

QUANTITY OF LIPASE REQUIRED TO ABOLISH STEATORRHEA

Normal pancreas

Normally, lipase is secreted early in the postprandial pe-

Table 3 Currently available United States Food and Drug Administration approved pancreatic enzyme preparations[1]

DRUG	Stregth Lipase USP	Preparation	Diagmeter\e	pH[1]	Cost per tablet (United States)	Cost per 1000 units
CREON®						
Creon 3000	3000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$1.18	$0.39
Creon 6000	6000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$1.30	$0.22
Creon 12000	12000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$2.32	$0.19
Creon 24000	24000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$4.56	$0.19
Creon 36000	36000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$7.90	$0.22
Pancreaze®						
Pancreaze 4200	4200	Capsule with enteric coated microtablets	2 mm	5.5	$0.92	$0.22
Pancreaze 10500	10500	Capsule with enteric coated microtablets	2 mm	5.5	$2.29	$0.22
Pancreaze 16800	16800	Capsule with enteric coated microtablets	2 mm	5.5	$3.68	$0.22
Pancreaze 21000	21000	Capsule with enteric coated microtablets	2 mm	5.5	$4.58	$0.22
Zenpep®						
Zenpep 3000	3000	Capsule with enteric coated beads	1.8-1.9 mm	5.5	$1.27	$0.42
Zenpep 5000	5000	Capsule with enteric coated beads	1.8-1.9 mm	5.5	$1.21	$0.24
Zenpep 10000	10000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$2.39	$0.24
Zenpep 15000	15000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$3.47	$0.23
Zenpep 20000	20000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$4.71	$0.24
Zenpep 25000	25000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$5.83	$0.23
Ultresa®						
Ultresa 13800	13800	Capsule with enteric coated minitablet	2 mm	5.5	$3.01	$0.22
Ultresa 20700	20700	Capsule with enteric coated minitablet	2 mm	5.5	$4.46	$0.22
Ultresa 23000	23000	Capsule with enteric coated minitablet	2 mm	5.5	$5.47	$0.24
Pertyze®						
Pertyze 8000	8000	Capsule with bicarbonate buffered enteric coated microsphere	0.8-2.2 mm	5.5	$1.99	$0.25
Pertyze 16000	16000	Capsule with bicarbonate buffered enteric coated microsphere	0.8-2.2 mm	5.5	$3.99	$0.25
Viokase®						
Viokase 10440	10440	Non-enteric coated			$2.92	$0.28
Viokase 20800	20880	Non-enteric coated			$5.76	$0.28

[1]pH at or above which enzyme is designed to release most of the enzyme based on the package insert.

riod and reaches a maximum within the first hour; the majority of fat digestion and absorption normally occurs within the proximal small intestine[22]. The ability to measure lipase activity led investigators to ask whether there was a best, appropriate, or minimum amount of lipase needed to correct steatorrhea. The available data are confusing in part because lipase units are often presented in different units, making direct comparisons difficult. Many basic and clinical studies use either international units (IU) or United States Pharmacopeia (USP) units. Commercial products in the United States are rated in USP units (1 IU = 3 USP units). We will provide the results whenever possible in USP units. When the units are not clear (as in some older papers) we will simply state the units as lipase units or provide the units name used for that study. The strength of current products ranges from 3000 USP units to 36000 USP units of lipase per dosage unit (*e.g.*, per capsule) (corresponding to a range of 1000 to 12000 IU) (Table 3). The amount of postprandial lipase secreted under normal physiologic circumstances has been estimated at between 9000 to 18000 USP units/min[22,23] Measurements from a patient with a pancreatic fistula suggested that a 60 kg man would produce 192000 Cherry-Crandall units[24]. Overall, the results of such studies depend on the experimental methodology and may explain the wide variation noted[25]. As noted previously, the pancreas has a tremendous reserve capacity, and perfusion studies have suggested that approximately 5% of normal output is the threshold to maintain normal fat absorption[26]. Other studies report somewhat higher amounts[10,27].
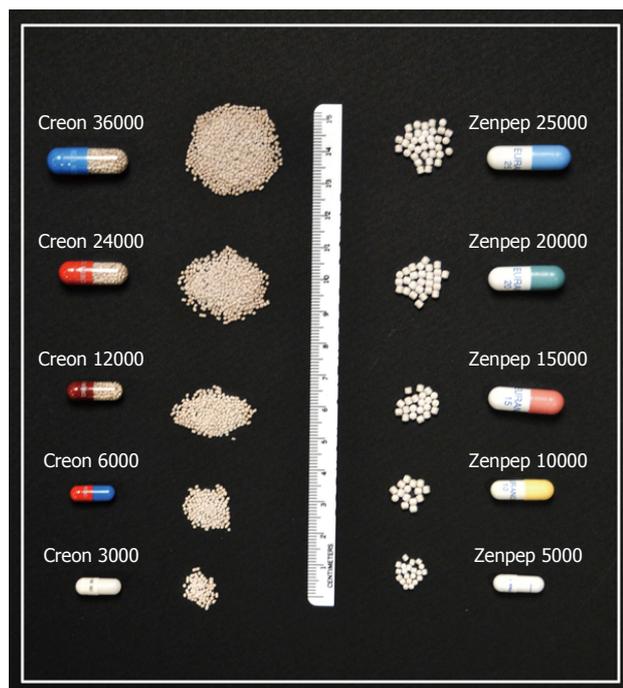
Figure 2 Pancreatic enzyme capsule size and contents increase as the pancreatic enzyme preparation dosage increases, suggesting that dose/unit increases are achieved by packaging the same basic pancreatic enzyme formulation into a larger capsule and/or larger beads.

Figure 3 Results of different pancreatic enzyme preparations, tablets, enteric-coated tablets, and capsule in adults with exocrine pancreatic insufficiency. Approximately 30000 USP units of lipase were given with meals. Steatorrhea was corrected in those with low acid secretion. From[28] with permission.

Figure 4 Effect of increasing the enzyme dosage on fecal fat excretion on a 100 gram fat diet. Enzymes were given 3 times per day with meals providing approximately 30000, 60000, or 120000 USP lipase units with each meal or as 18000 USP lipase units as enteric coated microspheres (*i.e.*, 3 tablets, 6 tablets or 12 tablets and 3 microsphere capsules with each meal). Each rectangle encloses the mean ± the standard deviation of the mean. The normal fecal fat is < 6 g/24 h. From[29] with permission.

Clinical results

Because it is difficult or even impossible to exactly simulate the normal integrated response of gastric emptying and pancreatobiliary secretion, estimates of the amount of lipase required to prevent steatorrhea are best determined clinically based on results of clinical trials. Trials using unprotected enzymes theoretically provide the most useful clinical measure, as they provide real time examples of pancreatic enzymes mixing and emptying with ingested nutrients coordinated with the function of the small intestine. However, interpretation of such studies is complicated by intragastric destruction of administered enzymes and by acidification of the duodenum, both of which can inactivate lipase and precipitate bile acids. Nonetheless, the available results probably provide our best estimates.

We performed studies with patients with varying degrees of acid secretory capacity and showed that we could abolish steatorrhea with approximately 30000 USP units of unprotected lipase given with meals (discussed in more detail in the section on the gastric pH barrier below). That study showed that a relatively small quantity of lipase was sufficient as long as the enzymes were able to mix with the meal and the lipase was not destroyed by gastric acidity (Figure 3)[28]. In a subsequent study with an enteric coated preparation, 2 of 6 patients experienced complete resolution of steatorrhea with only 18000 USP units of lipase with each meal when the enzyme was administered throughout the meal as enteric-coated microspheres (Figure 4)[29]. Overall, it seems reasonable to con-

clude that between 18000 and 30000 USP units of lipase per meal will result in resolution of steatorrhea, provided that lipase is delivered to the small intestine along with the nutrients and that low gastric and duodenal pH are not present. Achieving these coordinated events, however, to "deliver a sufficient amount of active lipase at the right place, *i.e.*, duodenum and proximal jejunum, and at the right time, *i.e.*, in parallel with gastric emptying of nutrients"[3] (Table 2) has proven difficult.

Gastric pH barrier

Lipase is irreversibly inactivated at a pH of 4 or less. Trypsin and the other enzymes are more acid stable but

A

B
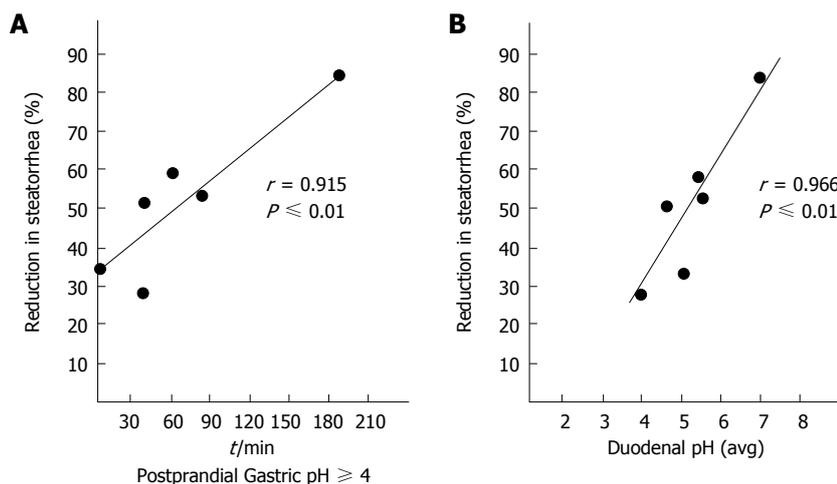
Figure 5 Correlation between percentage reduction in steatorrhea based on the median obtained with 30000 USP units of lipase given in tablets or capsules compared with the time of the postprandial gastric pH was > 4 (A) and in those same subjects compared with the mean post prandial duodenal pH (B). From[28] with permission.

$r = 0.915$
$P \leqslant 0.01$

$r = 0.966$
$P \leqslant 0.01$
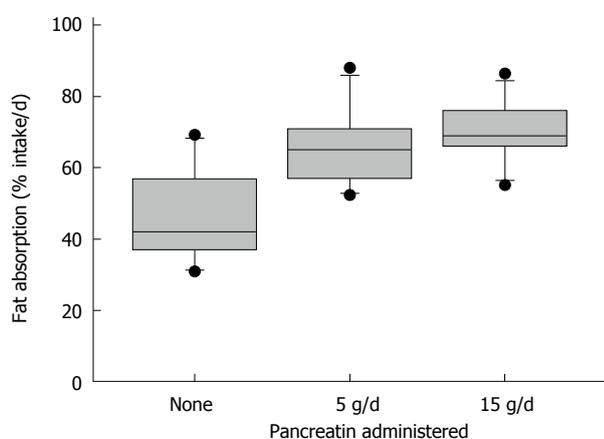
Figure 6 Results of a study comparing the response of enteric coated pancreatic enzyme in a cystic fibrosis patient population. Different doses of enteric coated pancreatic enzymes were taken four times daily immediately before meals and the corresponding average % fat absorption/d[11].

are also destroyed by pepsin in an acid environment[30,31]. Reliable enzyme therapy is therefore easiest to achieve in achlorhydric patients where the gastric pH barrier is absent. For example, we compared different enzyme formulations (2 tablet formulations and one capsule formulation produced by three different manufacturers, including one enteric coated tablet) in 6 patients who varied greatly in terms of their ability to produce acid[28]. The enteric coated tablet was effective only in one subject who also had hypo-/achlorhydria. We assessed the gastric barrier as the average time the gastric pH remained above 4 and the small intestinal pH barrier as the mean duodenal pH during meals. The effect of therapy on steatorrhea was almost identical for each individual subject (Figure 3) but varied between individuals with respect to gastric and duodenal acidity (*i.e.*, increasing acidity had a negative effect on reducing steatorrhea) (Figure 5)[28].

In subsequent studies with a different set of subjects, we examined whether the traditional approach of increasing the amount of unprotected enzymes would improve the effectiveness of therapy (in essence-was there a dose-response effect?)[29]. Doubling the amount of lipase

from approximately 30000 USP units per meal to 60000 USP units per meal did not provide an improvement in fat malabsorption (Figure 4). However, quadrupling the lipase dose to 120000 USP (*i.e.*, 12 tablets per meal) did result in improvement in fat absorption (*i.e.,* decreased fat loss) but in only 2 of the 4 subjects tested (Figure 4). Importantly, none of these subjects had resolution of steatorrhea. As noted previously, in another study with different subjects, administration of only 18000 IU of lipase/day as an enteric-coated microbead preparation resulted in resolution of steatorrhea in 2 of the 6 subjects tested (Figure 4)[30].

As unprotected enzymes likely mix well with the nutrients, their effectiveness depends more on acid secretion and gastric emptying than on the quantity administered[30,32-34]. The window of effective unprotected enzyme therapy is defined as the time between ingestion and the time at which the gastric pH falls below 4 which inactivates lipase. Gastric contents tend to layer with the lowest pH being concentrated at the periphery of the meal. Thus, any lipase within the bulk meal may be protected and remain active, but will be inactivated upon mixing with acid contents in the antrum during emptying into the small intestine. Overall, our results confirmed longstanding clinical experience that, although increasing the amount of enzyme administrated may result in an improvement in fat absorption, it generally will not consistently eliminate steatorrhea (Figure 6)[11,12,29,35].

GASTRIC EMPTYING AS A BARRIER TO SUCCESSFUL PANCREATIC ENZYME THERAPY

The initial barrier is the acidic gastric environment that can inactivate pancreatic enzymes. The enzymes also must also mix with the nutrients to be delivered together to the duodenum. The normal gastric antrum grinds and returns food to the body of the stomach. Most nutrients are emptied as small particles (< 1 mm) suspended within the liquid layer[36]. Depending on their size and density, enzyme microspheres may separate from bulk nutrients
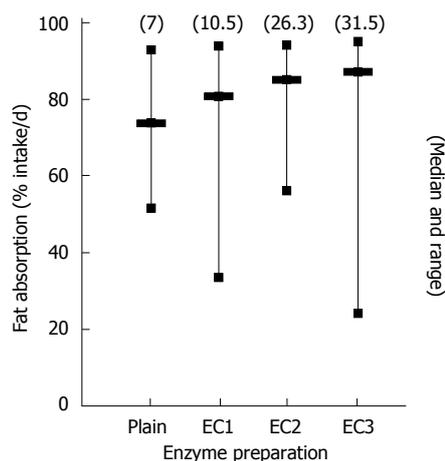
Figure 7 Randomized cross-over study in patients with cystic fibrosis and pancreatic insufficiency that compared plain uncoated enzymes (Pancrex V Forte *n* = 14) and 3 different enteric coated preparations [EC1:Pancreatin Merk, EC2: Creon and EC3: Pancrease (*n* = 19)] using the same lipase dosage. The median and range are shown of fecal fat absorption. With the numbers above the columns ( ) indicating the percent of patients with > 90% fat absorption. None reliably resulted in normalization of fat malabsorption[42].

and empty separately, thus impeding the interactions critical for digestion[37-39]. Normally, the stomach sieves and retains large particles until after the meal is emptied. This sieving occurs both in the proximal and distal stomach[36,37,40,41]. Currently available enteric coated enzyme beads vary with respect to enzyme content and diameter (*i.e.*, larger doses contain more units of enzyme per bead and may reach up to 2.5 mm in diameter) (Table 3). The dissolution and emptying characteristics of the different enzyme preparations and sizes remains unknown, as the FDA-requested studies have yet to be published. However, based on prior studies, each preparation is likely to have a different emptying profile. There is limited information available regarding the dispersion and emptying of enteric coated microspheres of different diameter and density, particularly in relation to fat malabsorption in humans. Comparative studies of 4 older preparations (Pancrex V Forte, Pancreatin Merk, Creon and Pancrease) showed differences in effectiveness, but it remains unknown whether the differences were primarily related to differences in the emptying of the beads or related to other factors (Figure 7)[42].

The ideal therapy is one that coordinates emptying of the meal and pancreatic enzymes. A significant proportion of ingested fat is emptied during the first hour of the meal, and normal physiologic lipase secretion is highest during this time[38,43-45]. However, enteric coated enzyme microbeads administered with meals tend to remain in the proximal stomach during the first hour, allowing a considerable proportion of fat to escape contact with enzymes and thus escape digestion[38,44]. Gastric emptying of enzymes and nutrients is better coordinated after the first hour, which is likely responsible for the improvement in absorption seen[38,44].

Overall, it is likely that a mismatch of emptying of fat and enzymes is a major contributor to the failure of

currently available microbead preparations to fully correct steatorrhea. Bruno *et al*[39] administered microbeads before meals and noted that they separated from the meal and tended to clump in the antrum, although some of the beads emptied even prior to the meal. This finding suggests that one approach to improving therapy is to optimize the timing of the administration of microbeads to reduce or eliminate periods of dissociation of emptying of fat and microbeads.

Although the FDA requested that companies perform studies regarding kinetics of enzyme release of approved products (namely, the when, where, and how much enzyme is released), none of the studies performed to date have yet to be published (*e.g.*, clinicalTrials.gov NCT00676702, Pancrease MT, Johnson and Johnson Pharmaceutical, NJ, United States; NCT00744250, NCT00749099 Pancrecarb MS16, Digestive Care, PA, United States; NCT00559052, Viokase 16, Axcan Pharma, Canada). We requested this and other information such as the median and range of fat absorption from each manufacturer; however, the manufacturers were unresponsive. Importantly, no head to head comparative studies of current FDA approved products from different manufacturers or different formulations of a single product are available. It therefore remains unclear how much, if any, interchangeability there may be between or even within products. It is also not known whether the source of porcine pancreatic enzymes used by different manufactures comes from one or a number of sources.

SMALL INTESTINAL PH BARRIER

Normal lipid digestion and absorption involves hydrolysis of triglycerides as well as solubilization of the products of digestion for subsequent absorption[46,47]. These processes are pH dependent and are disrupted when pancreatic bicarbonate secretion fails to neutralize acidic gastric contents and prevent lipase inactivation and precipitation of glycine conjugated bile salts. In some patients this low pH environment extends far down the small intestine and impairs both digestion and solubilization[13,46,48]. In addition, enteric coated microbeads are designed to dissolve only when intraluminal pH is 5.5 or higher and may not dissolve until reaching the distal small intestine or even the colon[27,33,49-54].

USE OF ANTACIDS AND/OR ANTISECRETORY DRUGS TO EXTEND THE HIGH PH WINDOW

Successful use of unprotected enzymes requires the ability to prevent or reduce inactivation of administered lipase by gastric acid. Antacids have been used for this purpose since the 19th century. More recently the strategy has shifted to antisecretory drugs; however, a combination of both may be the best option. The strategy to prevent inactivation of lipase differs from treatment of
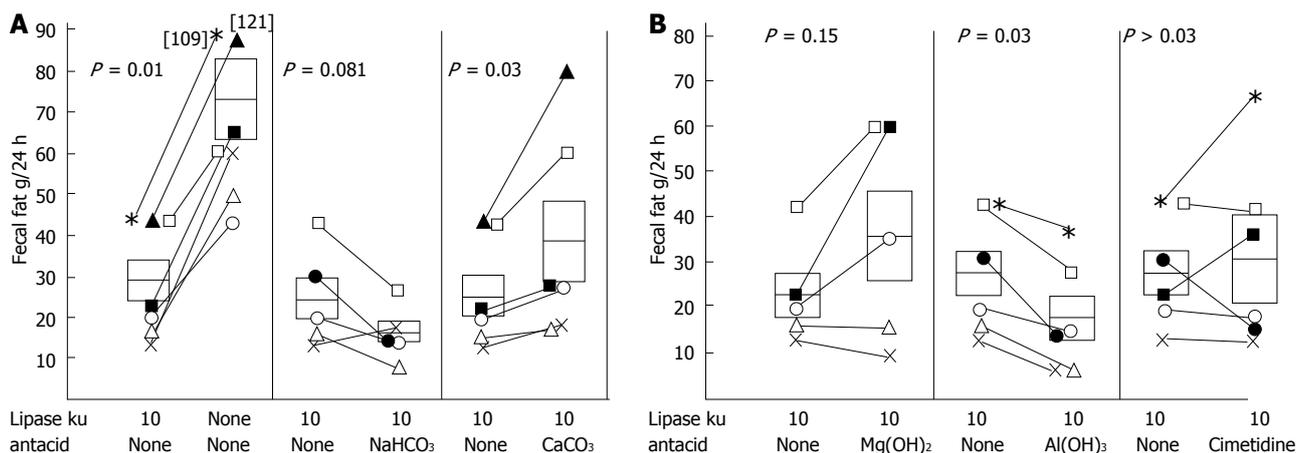
Figure 8 Effect of antacids and enzymes on the effectiveness of 30000 USP units of lipase per meal for the treatment of pancreatic steatorrhea. Each symbol represents a different patient. Sodium bicarbonate, magnesium aluminum hydroxide, aluminum hydroxide, or calcium carbonate were administered at the beginning and the termination of each meal. Cimetidine was given 30 min prior to the meal. From[58] with permission.
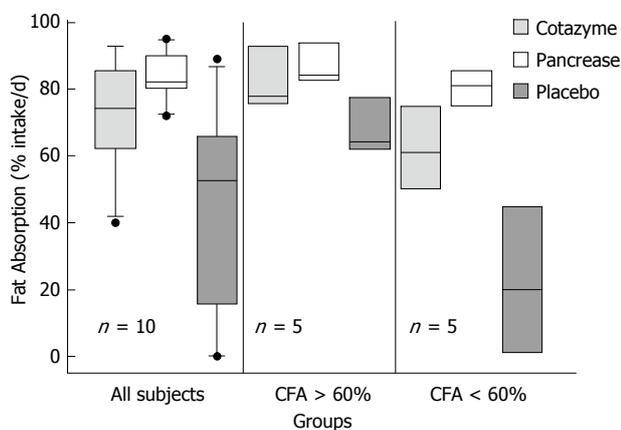
Figure 9 Randomized cross-over comparison of similar amounts of lipase administered as unprotected capsule (Cotazyme®) or enteric coated microspheres (Pancrease®) in cystic fibrosis patients with pancreatic insufficiency. Although the enteric coated preparation was better in those with the greatest degree of malabsorption, neither resulted in resolution of steatorrhea[61].

acid peptic disease. In peptic ulcer disease, the goal is to reduce gastric and duodenal acid load sufficiently to eliminate pain and heal the ulcer. In contrast, protection of lipase requires the much more stringent target that the gastric pH never fall to 4 or below (Table 2).

Early investigators reported only limited success in improving the effectiveness of enzyme therapy with co-administration of sodium bicarbonate or aluminum hydroxide[27,32,48,55-57]. We compared different antacids and the antisecretory drug cimetidine for their ability to improve the outcome of therapy with unprotected pancreatic enzymes[58]. We randomized subjects who had an incomplete response to 30000 USP units lipase per meal to receive commonly used doses of sodium bicarbonate (1.3 g; 12 mEq), aluminum hydroxide (30 mL; 57 mEq), magnesium-aluminum hydroxide (30 mL; 72 mEq), or calcium carbonate (1 g; 21 mEq). Each antacid was administered before and immediately after each meal (100

g fat per day)[58]. A final randomization was the 300 mg of the H$_2$-receptor antagonist, cimetidine, given 30 min before meals. Overall, cimetidine had no noticeable effect on fat absorption (Figure 8). In contrast, adjuvant therapy with either sodium bicarbonate or aluminum hydroxide resulted in a further reduction in steatorrhea (Figure 8). Strikingly, the highly effective antacids calcium carbonate and magnesium-aluminum hydroxide tended to reverse the beneficial effects of the enzyme therapy (Figure 8)[58]. Subsequent studies showed that the calcium and magnesium-containing antacids were effective in increasing intragastric and intraduodenal pH and improving the duodenal delivery of lipase and lipolysis[59]. However, both calcium and magnesium reacted with the fatty acids liberated to produce poorly soluble calcium and magnesium soaps that were poorly absorbed[59,60].

ENTERIC-COATING TO OVERCOME THE GASTRIC PH BARRIER

Using enteric coating is useful to bypass the gastric pH barrier and prevent gastric inactivation of pancreatic enzymes. The use of enteric coated microbead/spheres has resulted in more reliable results than had been obtained with enteric coated tablets (Figures 7 and 9)[42,61], but still fails to abolish steatorrhea for most patients[1,11,29,62-67]. The most common reasons given for an inadequate response to modern enteric coated enzyme therapy include: insufficient dosage, dissociation of the emptying of the microbeads and nutrients, premature opening of the microspheres in the stomach allowing intragastric destruction, long dissolution time which shifts the absorption sites distally, and rapid small intestinal transit which reduces mucosal contact time[33,36,37,43,44,51,68,69]. The benefits of modern enteric coated bead therapy appear greatest amongst those with the poorest responses to unprotected enzymes, most likely due to protection against rapid intragastric inactivation of unprotected lipase[33,42,49,61,66,70,71].
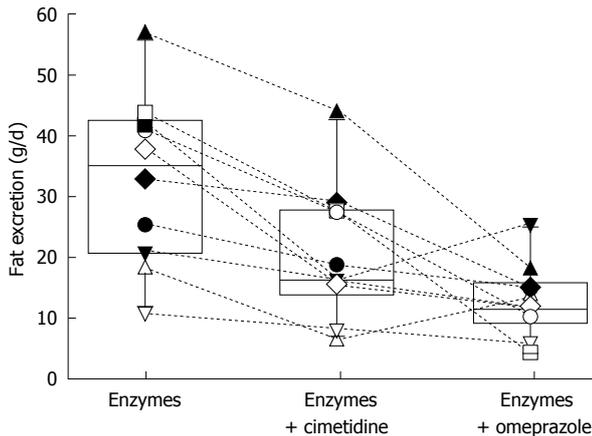
Figure 10 Box plot showing median and 25% and 75% and range for a randomized cross-over study comparing the effect of 1200 mg cimetidine or 60 mg of omeprazole on the effectiveness of pancreatic enzymes. Six tablets of unprotected enzymes (Cotazyme Forte® 36000 FIP units/meal) given 1/2 before meal and 1/2 during the meal). Both antisecretory agents improved outcome but neither reliably resolved steatorrhea. Data from[72].

Figure 11 Box plot showing median and 25% to 75% range for a randomized cross-over study comparing the effect of doubling the dose of pancreatic enzyme microspheres (Pancrease®) and the effect of omeprazole in patients with cystic fibrosis and pancreatic insufficiency. Enzymes were taken 1/2 just before and 1/2 after meals. Omeprazole 20 min before breakfast[67].

Attempts to improving the efficacy of enteric coated microbead enzyme therapy

Few studies have provided sufficient details to develop hypotheses for testing or insights into why success or failure occurs. The Mayo clinic group tested an early enteric coated microsphere formulation with and without adjuvant acid suppressive therapy[34]. They found that of the 2 of the 6 patients had complete resolution of steatorrhea. Both these patients had high acid secretion and the intragastric pH remained below 5.5. The remaining 4 patients with incomplete responses had higher gastric pH, suggesting that the poor responders may have released the enzymes in the stomach where they were subsequently inactivated when the pH fell[34]. Bruno *et al*[72] compared adjuvant cimetidine or omeprazole with an enteric coated microsphere preparation (Cotazyme Forte®). Normal fat absorption was not observed, but they reported a progressive improvement with increasing suppression of acid secretion (Figure 10), suggesting that antisecretory drugs may be useful adjuvants. A possible mechanism is sufficient reduction of acid secretion to increase the duodenal and small intestinal pH and thus enhance dissolution and effectiveness of enteric coated microbeads[72]. Data to support this hypothesis comes from Regan *et al*[34] who showed that following cimetidine administration, the duodenal pH remained above 6 for up to 200 min postprandial.

The pH burden is related to emptying of acidic gastric contents into the duodenum, which can respond poorly because of abnormal duodenal/pancreatic bicarbonate secretion. Antisecretory drug therapy is potentially most useful in those with gastric acid hypersecretion to reduce the duodenal acid load and allow acid neutralization despite impaired pancreatic secretion of bicarbonate. In one study, Heijerman *et al*[67] compared different doses of enteric coated pancreatic enzymes with and without omeprazole in patients with pancreatic insufficiency due
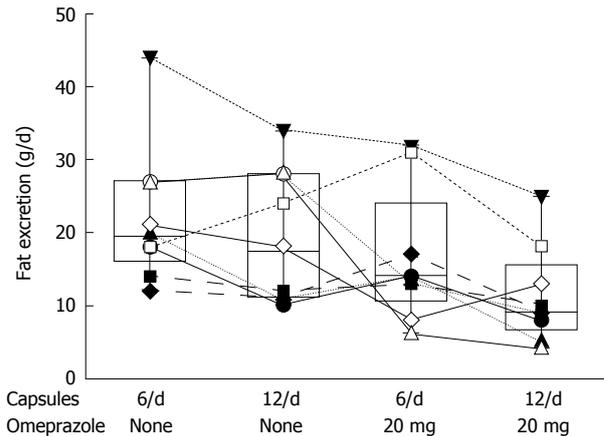
to cystic fibrosis with persistent steatorrhea. Increasing the dose of enzymes did not produce further improvement; however, increasing the enzyme dose and addition of omeprazole did (Figure 11). Overall, most studies with currently available preparations have not shown a consistent benefit for adding antisecretory therapy to enteric coated microbead therapy, except possibly among those with very poor response to enzyme therapy due to high gastric acid secretion[63,72-74]. Recent expert recommendations for use of pancreatic enzymes advise against the routine use of adjuvant proton pump inhibitor therapy[17].

Use of timing of dosing of pancreatic enzymes to improve outcome

In 1959, Jordan *et al*[12] compared 2 regimens in which 8 grams of unprotected enzymes (Viokase®) per day was given in 3 doses with meals or as 8 grams administered hourly from 8 a.m. to 7 p.m. (over 12 h). All 11 patients reduced their fecal fat excretion while taking pancreatic enzyme. Two patients failed to respond to the "with meals" regimen but experienced reductions in fat excretion with the hourly enzyme administration schedule. In contrast, Kalser *et al*[27] reported that administration of enzymes with meals (with adjuvant aluminum hydroxide) or on an hourly basis produced similar results. DiMagno *et al*[13] tested unprotected Viokase® (average of 10551 USP units lipase per tablet) administered either as eight tablets with each meal (2 tablets at the beginning, 4 tablets throughout the meal, followed by 2 tablets at the end of the meal) or as 2 tablets every hour for 4 doses at the onset of meal. In their study, irrespective of the dosing schedule, postprandial gastric pH fell below 4 after 40 min, the duodenal pH fell below 4 after 100 min, and less than 9% of lipase reached the duodenum.

Domínguez-Muñoz *et al*[73] performed a randomized three-way crossover study of 24 patients comparing 40000 USP units of Creon® enteric coated microbeads administered as 4 tablets before meals, 4 tablets just after

meals, or 4 tablets throughout meals (as 1 before, 2 during, and 1 tablet after meals). Enzymes were administered only with the 3 main meals of the day given immediately before or after meals or given throughout the meal (as described above, with 10000 USP units before the meal, 20000 USP units during the meal and 10000 USP units after the meal). The authors used the 13C-mixed triglyceride breath test as a surrogate for fat absorption. The percentage of patients who normalized fat digestion was 50%, 54% and 63%, respectively. There were no statistically significant differences and no definitive conclusions can be drawn.

Other issues related to enteric coating
In 1905, Chase wrote that "it is a well-known fact that pancreatin in substance, solution, or simple tablet, is soon rendered inert by the gastric juice when taken into the stomach. The recognition of this fact has led to the manufacture of pills and tablets of pancreatin coated with keratin, salol, *etc*. While such coatings do protect the ferment from the action of gastric juice, it is a question if they are dissolved early enough in the intestine to allow the pancreatin to be of any service in digestion"[15]. The issues raised by Chase in his 1905 review remain unanswered more than 100 years later. Patients with pancreatic insufficiency have alterations in gastro-intestinal motility as well as a reduction in bicarbonate secretion resulting in low intestinal pH, and both of these mechanisms may lead to unpredictable transit and dissolution of the different products. Current formulations are designed to release the enzymes when the pH allows their survival. However, failure to achieve an adequate pH at which dissociation of the coating can occur may delay the site of dissolution to the distal small intestine or even the colon[33,51]. Guarner *et al*[68] compared duodenal and ileal enzyme content of normal controls and patients with pancreatic insufficiency. When normal patients and patients with pancreatic insufficiency received placebo, there was a gradient of higher lipase enzyme activity in the duodenum and lower activity in the ileum. When given enzyme therapy as 5 enteric coated capsules each containing 8000 FIP lipase units (total of 40000 FIP lipase units), the gradient was reversed.

Current enteric coated preparations are available as microspheres or microbeads whose dissolution rate was established using standard FDA-approved *in vitro* dissolution tests. However, little is known about their dissolution or potential differences in dissolution rate *in vivo*, especially at different pH and different luminal environments. Available products generally contain microbeads/spheres of uniform size within a specific dose. However between products and even among products at different doses, the beads may differ in shape, size, and surface area and all of these physical characteristics may affect the kinetics of release of the enzymes[75]. *In vitro* studies such as those described by Löhr *et al*[75] on previously available products would be welcome, especially if the results were directly compared to the results of *in vivo* studies. As noted previ-

ously, any data the pharmaceutical companies have has been withheld. Even when or if these data are provided, to be fully useful they must include comparison studies in the same patients to determine the effects of size, shape, differences in coating, or other factors on bioavailability. Such studies may require support by agencies dedicated to exploration of important scientific question without a vested interest that might result in withholding the results.

There are a number of considerations regarding evaluation of the dissolution characteristics of enteric coated enzymes. The rate of dissolution of the enteric coated beads at any particular pH would likely be an important measure in determining where the enzyme is delivered in the small intestine. Aloulou *et al*[51] evaluated the dissolution times in relation to pH of three preparations including the non-coated Eurobiol 12500 and 2 enteric coated preparations, Eurobiol 25000® and Creon 25000®. Uncoated Eurobiol 12500 had essentially instant bioavailability. The half dissolution time of Eurobiol 25000® at pH of 5.2 was 19.2 min, contrasting markedly with Creon 25000® whose half dissolution time at pH of 5.4 was 49.2 minutes. Importantly, this *in vitro* study did not take into account the effect of other confounders such the presence of bile and other substances normally present *in vivo*. Overall bioavailability is likely determined both by the threshold pH of dissociation as well as the rapidity of dissolution.

We tested the dissolution time on Creon 24000®, Zenpep 25000®, and Ultresa 23000® in informal studies using ileal fluid obtained from a patient with an ileostomy. One capsule of each enzyme preparation was placed a 15 mL conical tube containing 7 mL of ileal fluid obtained from a patient with an ileostomy and then centrifuged. The pH was adjusted to approximately 7.5. The experiment was done using a water bath at 38 Celsius. The test tube was manually inverted 3 times every 1.5 min and visually inspected for onset and time to complete dissolution of the capsule. pH was measured at each time interval (Table 4). Each experiment was done in duplicate. The results suggest there are likely differences in dissolution time among the different products and possibly between the same product as different size microbeads. Formal *in vitro* and *in vivo* comparisons are warranted.

Because clinical assessment is a notoriously imprecise measure of effectiveness, a simple, non-invasive measure of overall effectiveness is needed to allow comparisons between and among products[76]. The ^{13}C mixed triglyceride breath test currently appears to be the best option[77,78] as it provides dynamic data regarding gastric emptying, dissolution, and effectiveness of enzyme therapy. It has the added benefit of being simple, non-invasive, inexpensive, and allows for efficient repeated testing of the same subjects. Using a validated breath test allows hypothesis testing and rapid evaluation of different combinations such as timing administration of enzymes in relation to meals, effects of dosage, acid suppression, *etc*. These overall conclusions could then be tested in a traditional clinical trial. Breath testing also allows for easy and effec-

Table 4 Dissolution time for pancreatic enzyme in ileal fluid				
Pancreatic enzyme	Initial pH	Start to dissolve (min)	Completely dissolved (min)	
Creon® 24000	7.73 pH	9.0	45.8	7.28 pH
Ultresa® 23000	7.52 pH	10.5	30.0	7.48 pH
Zenpep® 25000	7.60 pH	15.0	33.0	7.59 pH
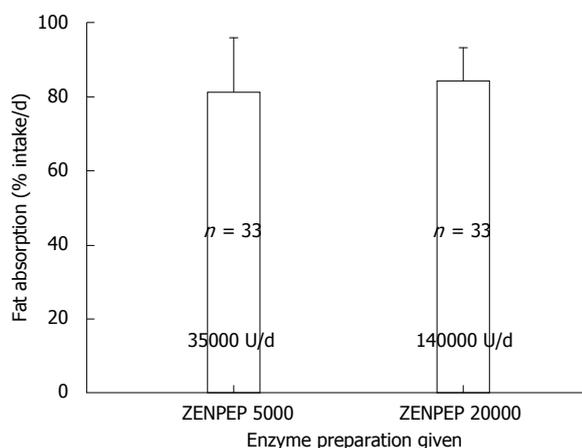
Figure 12 Effect of increasing the dose of enteric coated microbead therapy; seven 5000 USP unit tablets *vs* seven 20000 USP tablets (Zenpep®) on steatorrhea are shown (mean plus standard deviation). Increasing the dosage 4-fold resulted in no significant improvement in steatorrhea and did not result in correction of steatorrhea[4].

tive monitoring of therapy[77]. Unfortunately, despite being used in research for more than three decades, the test is not widely available outside of Europe and even there it is infrequently used.

APPROACHES TO THERAPY IN 2014-2015

Results with currently FDA approved enzyme preparations

The primary goal of enzyme therapy is to abolish steatorrhea. If this goal cannot be obtained, at the very least, one would like to achieve a coefficient of fat absorption >85% (*e.g.*, 15 g/d on a 100 g fat diet)[17,71]. The mean coefficient of fat absorption with modern enteric coated microspheres based on available data has typically been between 80% and 88% (*i.e.*, such that one third to more than one-half fail to achieve even this minimal desired outcome). Since at least the 19th century, the knee jerk response to inadequate results has been to increase the dosage. The "increase the dosage" strategy has carried over to the use of modern microbead therapy and the availability of high potency products[4,8,79] (Table 3). The published trials with currently available regimens were primarily designed to obtain regulatory approval for new products and for marketing purposes. The studies have therefore used similar protocols based on input from the FDA (http://www.fda.gov/downloads/Drugs/Guidance ComplianceRegulatoryInformation/Guidances/
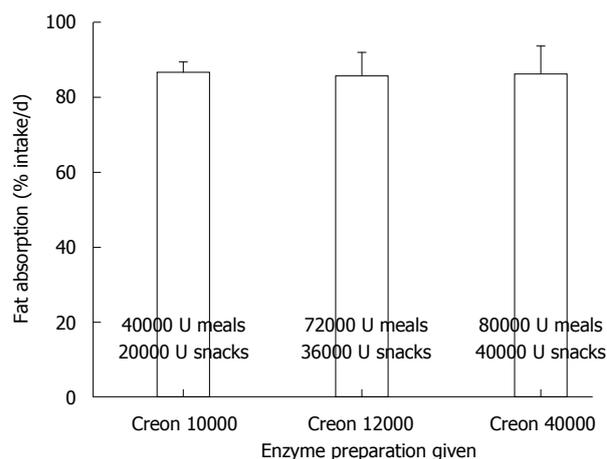
Figure 13 Summary data from 3 different randomized studies of different formulations of an enteric coated microbead product (Creon®). None of the formulations at the different doses given reliably resolved steatorrhea. Mean plus standard deviation of the different doses are shown[5,8,81].

ucm071651.pdf). These studies have been done well from a technical standpoint and used reliable methods for fecal collection and for analysis. The results are most often presented as the mean coefficient of fat absorption (CFA), which is calculated as [(fat intake - fat excretion)/fat intake] × 100 on a 72-h stool sample often collected in a controlled environment, plus the standard deviation. However, this presentation is of limited value to clinicians, as it does not provide definitive clinical data that would be useful in predicting clinical and symptom response, especially among patients with a previously unsatisfactory clinical response. For example, one would like to know the proportion of patients achieving a coefficient of fat absorption of at least 85%, as well as the median and range or 25%-75% values. Such data provide a clearer picture of what might be expected in clinical practice[42]. These data were requested from the manufacturers but not provided.

In some studies the patients may also not be representative. For example, Stern *et al*[80] included only patients who achieved at least 80% coefficient of fat absorption during a run-in phase on therapy, thus excluding the difficult to manage patients and improving the odds of an overall good outcome. In another study, approximately one-half of the subjects had minimal or no steatorrhea with placebo[4]. At least the data for the subgroup with significant steatorrhea was also provided separately in the outcome table[4]. Most trials have been relatively small because as they were powered only to detect a difference from placebo; however, the results may not extrapolate well to clinical practice. As shown in Figures 12 and 13[4,5,8,81] and Table 3, different formulations and lipase dosages have tended to provide similar results irrespective of the quantity of lipase administered. These results are consistent with the notion that only some of the lipase in the formulation was biologically available and overall was in excess of a threshold amount required to achieve the results reported. Importantly, these studies confirmed
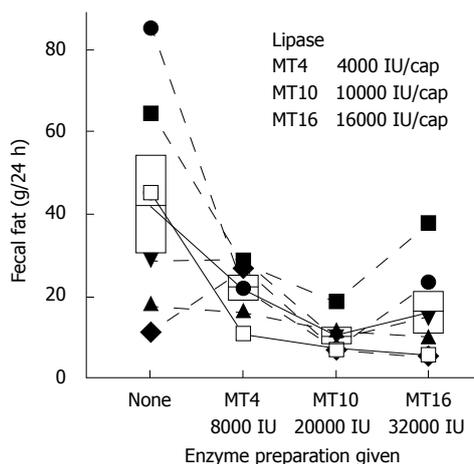
Figure 14 Effect of increasing the dosage of enteric coated microsphere preparation on fecal fat excretion is shown. Sorted by treatment groups and individual data for all subjects. Increasing the dosage from 8000 IU 4-fold (24000 to 128000 USP units) failed to show a clear dose response effect or to reliably resolve steatorrhea. The box shows the mean and standard deviation for each group. From[65] with permission.

Figure 15 Effect of acid suppression with 60 mg of omeprazole on effectiveness of enzyme therapy with an enteric coated microsphere preparation (Pancrease®). Comparison of 2 dosing regimens 10000 (2 capsule of 5000 USP Pancrease®) or 20000 USP (4 capsule 5000 USP Pancrease®) lipase units per meal. The results were the same and neither resolved the steatorrhea[82].

prior experience with enteric coated enzymes which also failed to show evidence of a dose response in terms of a reduction in steatorrhea[42,65,67,82] (Figures 7, 11, 14 and 15). Current products are priced in terms of dollars per units of enzyme (Table 3) such that the administration of more lipase than necessary serves only to increase cost to the patient without a corresponding increase in efficacy. A good example was a study that compared 7 capsules of Zenpep® 5000 (*i.e.*, 35000 USP units per day) a dose at which the authors expected "little or no effect on steatorrhea", with 7 capsules of Zenpep® 20000 (140000 USP units per day). The low and high doses produced similar outcomes (Figure 12)[4]. However, although the efficacy with high and low dose therapy did not differ, the cost of therapy per year was $11000 for high dose and $3000 for the equally effective low dose. These results confirmed that currently available products show (1) there is general lack of a dose-response effect; (2) increasing the dosage increases the cost more than the effectiveness; (3) a significant proportion of patients will still have clinically significant malabsorption despite enzyme therapy; and (4) a poor response to one dose generally signifies poor responsiveness to dose escalation.

One new preparation contains pancrelipase and sodium bicarbonate as a buffer to protect the enzymes and theoretically improve the pH in the small intestine (Pancrecarb®). It is called "highly buffered" although each capsule contains only 2.5 mEq of sodium bicarbonate. In clinical trials it was shown to be at best slightly better to not different from unbuffered capsules, and neither study achieved resolution of steatorrhea[83,84]. Currently, the FDA-approved Pertyze® is the only bicarbonate buffered pancreatic enzyme available. As noted above, studies of new concepts would probably be more efficiently initially evaluated using the ^{13}C-mixed triglyceride breath tests than through the use of expensive clinical trials.

Use of unprotected enzymes in the 21st century

An acid unprotected formulation of enzymes (Viokaze®) was recently FDA approved. While unprotected enzymes have limitations in relation to the relatively brief window in which the gastric pH is above 4, they may have a role in combination with enteric coated microbeads. In years past when *H. pylori*-associated atrophic gastritis was common, many adults had low acid secretion such that patients with pancreatic insufficiency often varied greatly in gastric secretory ability. In the modern era, *H. pylori* has become infrequent, and most adults exhibit normal acid secretion such that their intragastric pH falls to below 4 soon after eating and almost always within 60 min[54]. For these patients it is difficult to achieve or maintain an intragastric pH above 4 for a prolonged period using only antacids or antisecretory drugs. In the peptic ulcer era the goal of antacid or antisecretory therapy was to reduce acid output and thus the duodenal acid load. H_2-receptor antagonists typically reduce acid secretion by approximately 50%, which increases the average gastric pH for ulcer patients from approximately 1.4 to approximately 2, but increases the duodenal pH to above 4. Standard doses of proton pump inhibitors (*e.g.*, 20 mg of omeprazole) produce approximately a 90% reduction in acid secretion and an intragastric pH of 3 to 4[85]. A double dose (*e.g.*, 40 mg of omeprazole) provides 99% inhibition of acid secretion with narrow confidence intervals but will not reliably maintain the pH at 6 or above (which is the rationale for continuous infusion proton pump therapy in treatment of upper gastrointestinal ulcer bleeding)[85].

Studies of intragastric pH during meals have shown that the intragastric pH rapidly increases to the approximate pH of the meal, typically about pH 5, which stimulates the stomach to secrete acid maximally[54]. Initially, secreted acid is largely consumed by the buffering capacity of the meal such that average volume in the stomach remains relatively constant despite emptying. By 1 h, the in-

tragastric pH falls to approximately 3, resulting in down-regulation of acid secretion allowing gastric emptying to exceed secretion such that the intragastric volume and the pH to continue to fall[86-91]. In normal subjects, one can expect the intragastric pH to fall below the threshold for lipase destruction between 30 min and one hour after eating. The longer the acid secretory rate is suppressed, the longer the lipase can remain active. In peptic ulcer disease, the recommendation was to administer antacids 1 and 3 h after meals in order to reconstitute the buffering capacity of the meal and achieve the maximum benefits for treatment of peptic ulcer disease. When used as an adjuvant to enzyme therapy, the goal is to maintain the pH above 4 or above for as long as possible in order to prevent inactivation of lipase.

pH is measured on a log scale such that each unit of change signifies a 10-fold change in acid concentration. Thus, a pH of 1 is equal to 100 mEq/L and a pH 6 equals 0.001 mEq/L. Parietal cells secrete acid at a high concentration (*e.g.,* 140-160 mEq/L); hence only a few active parietal cells secreting a small amount of concentrated acid can drop the pH below 4 and inactivate lipase[85]. Since high intragastric pH stimulates the stomach to secrete maximally, it is practically impossible to provide sufficient sodium bicarbonate or aluminum hydroxide to reliably maintain the intragastric pH above 5. However, the combination of an antisecretory drug to inhibit parietal secretion, coupled with an antacid to increase the pH and neutralize the small amount of acid secreted after inhibition of the majority of parietal cells, should be effective. Sodium bicarbonate is probably the ideal antacid as it is "natural," widely available in 325 mg (4 mEq) and 650 mg (8 mEq) tablets, and cheap. Although the ideal strategy remains to be determined experimentally, we recommend use of a proton pump inhibitor such as 40 mg of omeprazole daily along with 650 mg sodium bicarbonate tablets administered whenever unprotected enzymes are administered (*i.e.*, 1 tablet 2 or 3 times with the enzymes during the meal) and 1 and 2 h after meals. Current technology using the Smart Pill®[92] or Bravo®[93] to measure pH in the stomach and duodenum should rapidly identify the ideal timing and dosage of administration of the sodium bicarbonate.

Use of unprotected and enteric-coated enzymes in combination

Another approach to improve the results of enzyme therapy is to take advantage of the benefits of both unprotected and enteric coated formulations. Unprotected enzymes mix well with the meal and initially provide high duodenal lipase activity and fat digestion. However, depending on the acid secretory ability of the patient, when the gastric pH falls below 4, lipase will be inactivated providing a pattern of "effective early-ineffective late" therapy[32,33,51]. This pattern can be overcome by inhibiting acid secretion and using antacids to raise the pH to extend the duration of high pH gastric contents.

The pattern of effectiveness of enteric coated beads is one of "ineffective early - effective late". Combining the two approaches by starting therapy with unprotected enzymes followed by coated formulations would theoretically achieve a pattern of "effective early and effective late" and provide enzymes in parallel with gastric emptying of nutrients. We previously recommended this approach based on our experience[94]. The concept is supported and was given a firm physiologic basis by the exquisite studies by Gow *et al*[32] and Delchier *et al*[33] who used gastric and duodenal intubation to evaluate duodenal pH, enzyme and bile acid concentrations, and intraluminal digestion combined with fat balance studies. Meyer *et al*[37] also recommended the combination of unprotected and coated enzymes based on their elegant studies of emptying of enteric coated microbeads. To our knowledge no one has taken up the challenge of further investigating the combination approach, possibly because the recent focus has been on obtaining regulatory approval for new products rather than optimizing their effectiveness. More efficient use of available products would also require less enzyme and thus lower sales. The recent availability of an approved uncoated product (Viokaze) now makes testing the hypothesis possible.

Putting it all together

Based on perfusion studies and on theoretical grounds it has been suggested that 25000 to 50000 USP units of lipase should be administered per meal to achieve normal fat digestion and absorption[22]. As shown above, experience with pancreatic enzyme therapy with individual patients has shown that 18000 to 30000 USP lipase units per meal is probably the minimum needed for complete resolution of steatorrhea. Clinical trials with patients always trump laboratory experiments, and theoretical models and trials are needed to test and confirm hypotheses regarding most efficient use of enzymes. The one common feature of studies that has shown complete correction of steatorrhea is the presence of active lipase in the intestines for long periods, either because of the administration of unprotected enzymes or dissolution of enteric coated products in the stomach and their continued activity because the pH remained high[13,28,33]. The enteric coated product studied by Delchier *et al*[33] (Eurobiol 25000®) was very slow to dissolve after it reached the small intestine such that the amount of lipase measurable at the ligament of Treitz was similar to that following placebo. In contrast, those with high intragastric pH and rapid gastric emptying had high levels of intraduodenal lipase as well as intraduodenal absorption of triglycerides. Because a significant proportion of fat is emptied during the first 30 min of the meal, it is critical to provide exogenous lipase during that period. Potential approaches to solving this problem include: (1) the use of antacids and antisecretory drugs to prevent intragastric acidification; (2) administration of uncoated enzymes and possibly some sodium bicarbonate at the start of the meal; or (3) identify a strategy of emptying enteric coated products in the earliest portion of gastric emptying (for example,
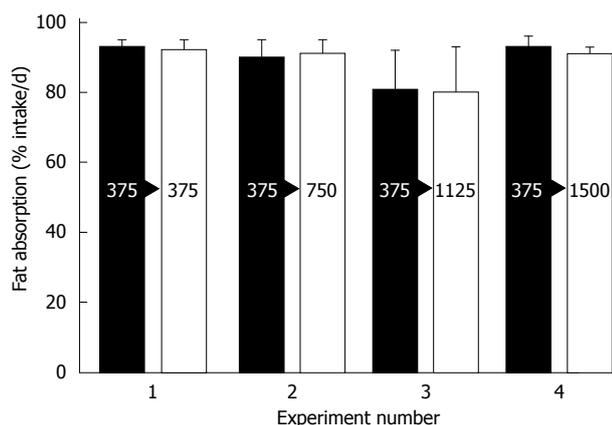
Figure 16 Data from 4 studies in children with cystic fibrosis comparing 375 USP lipase units/kg/meal to higher doses for the effect on steatorrhea. The results did not show a consistent effect on increasing the lipase dosage of an enteric coated preparation (Pancreaze®). Mean plus standard deviation are shown[79].

administer them before and during the meal). The dissolution characteristics of enteric coated products need further evaluation to examine when, where, how rapidly, and how completely the enzymes are released, and how these data relate to their clinical effectiveness.

Similarly, further studies are needed to address which changes in the timing of administration of pancreatic enzymes best coordinate pancreatic enzymes with emptying of gastric contents. For example, in three recent reviews the recommendations vary from 50% at the beginning of the meal and 50% at mid-meal[95], to during or immediately following the meal[96] and 25% with the first bite, 50% during the meal and 25% with the last bite[97]. From the available data and the data showing that a considerable amount of fat is emptied in the first hour, it is prudent when using enteric coated microbeads to start therapy just before the meal so that some microbeads are emptied during the first hour, then distribute the remaining enzymes throughout the meal. Those with hyperacidity may also benefit from adjuvant antisecretory therapy to reduce the duodenal acid load. However, it may not be possible to find an ideal schedule if one is restricted to using only enteric coated microbead therapy. Below we will discuss the available experience with currently approved therapies.

It has been known since the earliest days of pancreatic enzyme therapy that the patients who reliably experience good response are those with limited or no acid secretion. While the research focus has long been on duodenal lipase levels[22] one must now also consider how much and whether intragastric lipolysis due to the exogenous lipase contributes to the outcome. It should be clear that we have moved beyond the current "better than placebo" era of research aimed at obtaining regulatory approval for commercial products, and now need to focus on understanding how to reliably provide therapy and how to best use the available products.

More is not better using modern formulations

As a general rule for both unprotected and enteric-coated beads, the effect on steatorrhea is not directly related to the amount of lipase administered (namely, that after a threshold response, any further increase in the amount of enzyme given provides little or no additional benefit). This phenomenon has resulted in misinterpretation of many studies. For example, consider an experiment where the same dose of lipase is given using two different formulations (*e.g.*, 10 capsules are compared to 1 of another) with both formulations providing the same quantity of lipase. If both produce the same reduction in steatorrhea, the investigators would be tempted to conclude that one could use the formulations interchangeably, provided that the same quantity of lipase was administered. However, if they had included controls with one-half and with double the quantity of enzyme, they would likely have achieved the same result. This trap was revealed by studies examining whether there was a lipase dose - fecal fat responses (*e.g.*, Figures 12-16)[4,5,8,65,79,81,82]. For example, administration of 8000, 20000 or 32000 units of lipase using three different preparations of an enteric-coated commercial product produced no consistent change in fat malabsorption[65] (Figure 14). Figures 12, 13, 15, and 16 show more recent examples with a variety of enteric-coated products[4,5,8,81,82,98]. Figure 16 is especially revealing: in this study 4 subjects per group (children with cystic fibrosis) received therapy with 375 units of lipase/kg per day and then were given a different dose of 375, 750, 1125, or 1500 units/kg per day[79]. Clearly, the results with increasing to higher doses were almost identical.

Marketing strategies of companies selling pancreatic enzymes include attempts to link the amount of lipase required to fat intake and suggest that providers or patients increase the dosage in response to an unsatisfactory clinical response. Except for the low dosage products (which are priced about twice as high), enteric-coated pancreatic enzymes are currently priced between $2 and $4 per 10000 lipase units (Table 3). The lack of studies showing "more is better" and lack of head-to-head comparisons makes choice of therapy a matter of judgment.

Adding microspheres to food or putting them down feeding tubes

Enteric coated products to be taken orally are designed to dissociate when the pH is 5.5 or greater. The Cystic Fibrosis Foundation recommendations are consistent with the current package inserts: for infants and patients that are unable to swallow, recommended administration is to open the capsules and sprinkle its contents onto soft food mixtures with pH of 4.5 or less (*e.g.*, applesauce). The recommendation is based on theory rather than analysis of interaction of the enteric coating with complex formulations such as food. Sackman *et al*[99]. addressed the issue of mixing enteric-coated pancreatic enzymes with various food contents at various pH. They incubated en-

Table 5 Data needed to understand how to use new enzyme formulations

Results of all studies should not be withheld but should be published and/or placed on Clintrials.gov within 1 yr of completion

Trial data should provide the primary efficacy endpoint (*e.g.*, coefficient of fat absorption) as mean, standard deviation, median, range, and proportion with coefficient of fat absorption > 90% as well as proportion with coefficient of fat absorption < 85%

Gastric emptying of enteric coated pellets studied for all products are needed and the data should be published and/or placed on Clintrials. gov within 1 yr of completion

Kinetics of dissolution of enteric-coated microbeads in intestinal fluid or simulated intestinal fluid are needed and should include data pH's starting at approximately pH 5 through 7 at increments (*e.g.*, approximately 0.2 pH units)

Table 6 Recommended clinical trials

Head to head comparisons of different formulations within a product line as well as between commercial products

Comparative trials using different patterns of administration in relation to meals of enteric coated products (*e.g.*, before and during)

Studies combining unprotected and enteric coated preparations

Studies of unprotected preparations combined with maintenance of the intragastric pH constantly above 4

Initial pilot studies using ^{13}C-mixed triglyceride breath testing to test proof of concept may be the most efficient means of identifying which studies to test in human clinical trials

teric coated enzymes in saline, various food products with pH ranging from 5.6 to 6.5, and applesauce with pH of 3.4 and measured dissolution time as a surrogate for the integrity of the enteric-coating. Trypsin activity was used as a surrogate for lipase release. Among the foods tested, only applesauce reduced the integrity of the enteric-coating[99]. That study was conducted in 1982 with an older formulation but showed that theory is always subject to confirmation by experimentation. Studies with newer formulations are needed. Until that time it is likely that mixing with any food would be safe, although applesauce should probably be avoided. Shlieout *et al*[100] in an *in vitro* study mixed Creon 12000® in various baby foods with pH 4.5 or less to study use of pancreatic enzyme activity after passing it through various G-tubes. They found that the 16F Kimberly-Clark MIC-KEY tube was the smallest diameter tube that allowed passage of all food mixtures without clogging. Using tubes from other manufactures, they found that only 18F and larger tubes were able to pass all food content without clogging. All preparations retained 89.9% to 96.9% of the expected lipase activity. Nicolo *et al*[101] published 4 cases of patients dependent on enteric feeding and pancreatic enzyme supplementations. They reported that mixing pancreatic enzyme in all vehicles, including saline, applesauce, and fruit juices resulted in clogging of the tube; however, mixing the pancreatic enzyme in 8.4% solution of bicarbonate was effective. Interestingly, the combined use of pancreatic enzymes and bicarbonate is a common method used to unclog feeding tubes[102].

Recommended therapy

For the average patient, we recommend three, approximately 10000 USP units of lipase containing enteric coated microbead capsules/tablets per meal and one with snacks (*e.g.*, approximately 40000 USP units for an adult). The first dose is given before meals and the others during the meal. Following an unsatisfactory response one might consider adding approximately 20000 units lipase during meals. There are no data that increasing the dosage further increases effectiveness and is likely "beating a dead horse". Instead one should consider changing to a product with different characteristics (*e.g.*, from a microsphere to a minitablet), adding a unprotected enzyme product at the start of the meal, and/or adjuvant therapy with an PPI and/or sodium bicarbonate. As noted previously, one-third to more than one-half of patients will require therapy to be individualized. One should also consider the possibility of a second cause of malabsorption such as celiac disease or bacterial overgrowth. Treatment success should be assessed clinically and whenever available by an estimate of fat absorption. Longer term success should also be monitored in terms of maintenance of normal levels of fat soluble vitamins.

CONCLUSION

Hopefully, the current era of studies primarily targeted to obtaining FDA approval and marketing new products will soon transition into an era focusing on overcoming the remaining barriers that have limited the overall effectiveness of pancreatic enzyme therapy. In many ways we have not progressed beyond what was known in the 1980's. There are many options that potentially would improve current therapy and we have outlined a number of possibilities (Tables 5 and 6). A number of options need further testing, including the effects of combining unprotected enzymes (given with the first few bites and/or with sodium bicarbonate to buffer residual acid) in combination with enteric coated enzymes given throughout the meal. Hopefully comparative studies and studies of gastric emptying and dissolution of each formulation during normal meals will be done, and that results of those studies will be published in a timely manner.

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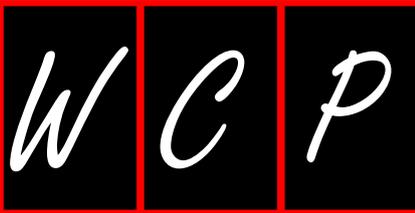
Endoscopic ultrasonography-guided endoscopic treatment of pancreatic pseudocysts and walled-off necrosis: New technical developments

Barbara Braden, Christoph F Dietrich

Barbara Braden, Translational Gastroenterology Unit, Oxford University Hospitals NHS Trust, Oxford OX3 9DU, United Kingdom

Christoph F Dietrich, Med. Klinik 2, Caritas Hospital, Uhlandstr. 7, 97980 Bad Mergentheim, Germany

Author contributions: Braden B wrote the paper; Dietrich CF critically revised the manuscript.

Correspondence to: Barbara Braden, Professor, Translational Gastroenterology Unit, Oxford University Hospitals NHS Trust, Headley Way, Oxford OX3 9DU,
United Kingdom. braden@em.uni-frankfurt.de

Telephone: +44-18-65228760 **Fax:** +44-18-65228763

Abstract

In the last decades, the treatment of pancreatic pseudocysts and necrosis occurring in the clinical context of acute and chronic pancreatitis has shifted towards minimally invasive endoscopic interventions. Surgical procedures can be avoided in many cases by using endoscopically placed, Endoscopic ultrasonography-guided techniques and drainages. Endoscopic ultrasound enables the placement of transmural plastic and metal stents or nasocystic tubes for the drainage of peripancreatic fluid collections. The development of self-expanding metal stents and exchange free delivering systems have simplified the drainage of pancreatic fluid collections. This review will discuss available therapeutic techniques and new developments.

Core tip: Endoscopic ultrasonography (EUS)-guided drainage of pancreatic pseudocysts and walled-off necrosis has become an established less invasive management of these difficult to treat complications of acute and chronic pancreatitis. New developments such as forward-viewing echoscopes and exchange-free delivery systems for the insertion of stents and drainages have simplified the technically challenging procedure. Specially designed self-expanding metal stents aim on improved drainage of the cyst content. This article reviews new EUS-guided techniques and their indications.

INTRODUCTION

Peripancreatic fluid collections frequently occur in the context of acute and chronic pancreatitis. Fortunately, more than 50% will resolve spontaneously and, therefore, a conservative expectant approach is often the right clinical decision.

The updated Atlanta classification[1] tries to overcome the existing confusion in reporting morphologic features and complications of pancreatitis. The revised definitions differ between acute peripancreatic fluid collections associated with acute interstitial oedematous pancreatitis and acute necrotic collection occurring in necrotising pancreatitis in the acute phase. On the other hand in the late phase after more than 4 wk, the classifications describes pancreatic pseudocysts developing from interstitial oede-

matous pancreatitis or walled-off necrosis resulting from necrotizing pancreatitis (Table 1).

Only superinfected or clinically symptomatic pseudocysts should be considered for interventional treatment. Infection, pain, malnutrition or compression of biliary, intestinal or vascular structures might present an indication for endoscopic or percutaneous drainage. The size of the cyst alone should not influence the decision for interventional treatment.

Apart from external and transmural endoscopic drainages, some pseudocysts with communication to the pancreatic duct might be suitable for transpapillary drainage *via* endoscopic retrograde pancreatography (ERP). It is also possible to combine percutaneous and transmural drainages to allow frequent flushing through the external drain; similar techniques also exist for necrosectomy (hybride necrosectomy)[2,3].

Infected pseudocysts, pancreatic abscesses and infected necrosis often require drainage. Preferably, the intervention should be delayed to at least 4 wk or longer - if possible - after disease onset to allow demarcation and liquidification of the necrosis. Despite all enthusiasm for new technical developments and minimal invasive techniques, we should not forget that conservative management with antibiotic therapy alone can also result in a good outcome in selected, clinically stable patients[4-6]. Therefore, ideally, all decisions for intervention and when to intervene should be discussed in a multidisciplinary team.

ENDOSCOPIC ULTRASONOGRAPHY GUIDED PSEUDOCYST DRAINAGE

Even in large bulging pseudocysts, the endoscopic ultrasonography (EUS) guided drainage is superior to the purely endoscopic approach as the puncture of vascular structures can be avoided by Doppler sonographic visualization[7,8].

The puncture of the cyst is usually performed using a 19G needle under endosonographic view. Cyst content can be aspirated for biochemical analysis [amylase or lipase, carcinoembryonic antigen (CEA)], gram stain, culture and cytology. Through the lumen of the needle a 0.035" guide wire can be advanced until it curls up in the cyst which adds stabilisation of the position and access by forming anchoring extra loops in the cavity[9-11].

Enlargement of the newly created ostium can be achieved by balloon dilatation over the guide wire. Alternatively, the canal can be widened using a cytostome and diathermy. The cytostome also allows the direct puncture of the cyst as it contains an integrated needle knife catheter which can be used instead of the 19G needle but lacks stiffness. The outer metal ring of the cytostome allows application of diathermy and the creation of a 10 Fr channel.

Stents or nasocystic tubes can be placed over the guide wire. Usually, pigtail stents are preferred due to a

Table 1 Modified according to the updated Atlanta classification for definition of peripancreatic fluid collections based on contrast enhanced computed tomography criteria[1]

	Acute phase < 4 wk	Late phase > 4 wk
Interstitial oedematous pancreatitis	Acute peripancreatic fluid collection (homogenous with fluid density, no definable wall, no necrosis, adjacent to pancreas, not intra-pancreatic)	Pancreatic pseudocyst (well circumscribed, usually round or oval, homogenous fluid density, no debris, well defined wall)
Necrotising pancreatitis	Acute necrosis (heterogenous and also non-liquid density, no definable wall, intra- and/or extrapancreatic location)	Walled-off necrosis (heterogenous and also non-liquid density, well defined wall, completely encapsulating, intra- and/ or extrapancreatic location)

Table 2 Equipment for endoscopic ultrasonography-guided pseudocyst drainage

Ultrasound processor
Linear array echoscope with 3.8 mm instrument channel
19 G EUS needle or cytostome and HF generator
Stiff guidewires (*e.g.*, Jagwire™)
Dilatation balloon catheter
Pigtail prosthesis (*e.g.*, 10 F)
Fluoroscopy optional

reduced dislocation rate. The insertion of two or more stents is desirable to improve cyst drainage and to prevent occlusion of stent and ostium as the cyst content then also empties through the gaps between the stents. Direct insertion of two guide wires into the cyst through balloon catheter or cytostome before insertion of the first stent facilitates the placement of the second stent.

Recently, the so-called multiple transluminal gateway technique has been reported for treatment of walled-off necrosis. This method requires the EUS-guided creation of two or three transmural tracts between the necrotic cavity and the gastrointestinal lumen. While one tract is used to flush saline solution *via* a nasocystic catheter, multiple stents in the other tracts are deployed to facilitate drainage of necrotic contents. This method was superior compared to the conventional single tract technique and might avoid the need for endoscopic debridement or open surgery in some cases[12].

The EUS-guided stent placement can be performed by endosonographic and endoscopic visualization only, however, fluoroscopy is often helpful, especially as the endosonographic view can become difficult after the cyst puncture and during stent placement (Table 2).

It can be challenging to discriminate pseudocysts from benign and malignant cystic neoplasia. Morphological criteria including vascularized septa and solid nodules in the wall of the cyst are indicators for a cystic neoplasia. Contrast enhanced ultrasound has a major impact for the diagnostic workflow of pancreatic cystic lesions[13,14]. The biochemical analysis of the cystic fluid including amylase and the CEA has proved to be helpful

Figure 1 Endoscopic ultrasonography guided placement of a transgastric metal stent allows endoscopic access into the necrotic cavity for endoscopic debridement. A: Gastric end of the covered transmural stent; B: Endoscopic view within the stent showing blocking necrotic material; C: Endoscopic view of the necrotic cavity after passage of the endoscope through the metal stent (two weeks after flushing *via* a nasocystic tube).

in the differential diagnosis between mucinous tumours (IPMN and mucinous cystadenoma with high CEA) and pancreatic pseudocysts (high amylase and low CEA).

ENDOSCOPIC NECROSECTOMY

In case of extensive necrosis, it can be necessary to extract the necrotic tissue from the walled-off cyst in order to induce the healing process. This requires the creation of a large caliber transmural orifice between the gastric or duodenal and the cystic lumen which allows the passage of a standard endoscope into the cystic cavity. In 2000, Seifert reported the first three cases of endoscopic debridement of infected retroperitoneal necrosis[15]. Since then multicenter studies have proven reduced mortality

and morbidity for endoscopic necrosectomy compared to the open surgical approach[16].

Conventional endoscopic snares and dormia baskets can be used to carefully extract the necrotic material into the stomach or duodenum. Usually, many repeated endoscopic sessions (on average 4 in a recent meta analysis[17]) are necessary to mobilise the necrotic tissue completely. Complications such as perforation (4%) and bleeding (18%) can occur during endoscopic necrosectomy. Particularily the bleeding can be horrendous because often large vessels transverse the cysts or necrotic cavity. Such procedures should only be undertaken by experienced endoscopic interventionalists in high volume centers that have back up by skilled hepatobiliopancreatic surgeons and interventional radiologists. In a recent meta analysis from 14 studies, more than 80% of patients with walled-off necrosis could be successfully treated by endoscopic necrosectomy alone; this was associated with 6% mortality and a complication rate at 36%[17].

Use of carbon dioxide insufflation is mandatory to avoid air embolism.

NEW DEVELOPMENTS

Antegrade echoscope
An echoendoscope with an antegrade view of 120 degree and 3.7 mm instrument channel has been designed by Olympus (TGF-UC260J) to improve the endoscopic orientation after the initial puncture facilitating further interventional steps such as dilatation or stent inserting. The straight instrument channel allows better instrument control due to reduced resistance. Compared to conventional linear echoscopes the antegrade type has curved linear array with a much shorter rigid portion at the tip and a capability to angulate the tip up to 180 degree which improves manoeuvrability and *e.g.*, enables retroflexion in the fundus. An auxillary water channel flushes away blood and turbid cyst contents for clear endoscopic views.

First studies including a randomized controlled multicenter trial have shown promising results compared to conventional longitudinal echoendoscopes[18-20].

New self-expandable metal stents
Usually, metal stents are not required to drain pseudocysts containing clear fluids but for infected cysts and walled-off necrosis a long term securing of a large diameter cyst opening by a metal stent can be helpful to allow drainage of larger particles and repeated direct endoscopic debridement (Figure 1). During the last years, self-expandable metal stents (SEMS) have been adapted to the needs of EUS guided cyst drainage. Large flanges should prevent dislocation of the stent, particularly feared is the migration of the stent into the necrotic cavity. The stents are covered to avoid leakage between cyst and stomach wall, it also prevents ingrowth and enables easy removal at a later timepoint. The self-extendable metal stents designed for drainage of pancreatic fluid collections and

Table 3	Covered self-expandable metal stents for endoscopic ultrasonography guided pancreatic cyst drainage					
Stent	**Company**	**Length (mm)**	**Internal diameter (mm)**	**Maximal flange diameter (mm)**	**Delivery device length (mm)**	**Delivery device diameter (Fr)**
Axios[TM]	Xlumena	10	10 or 15	21 or 24	1460	10.8
Aix[TM]	Leufen	30	10 or 15	25	2300	10
Nagi[TM]	Taewoong	10 or 20 or 30	10 or 12 or 14 or 16	22 or 24 or 26 or 28	1800	10.5
BCF[TM] Hanaro	M.I. Tech	30 or 40	10 or 12	25	1800	10.2
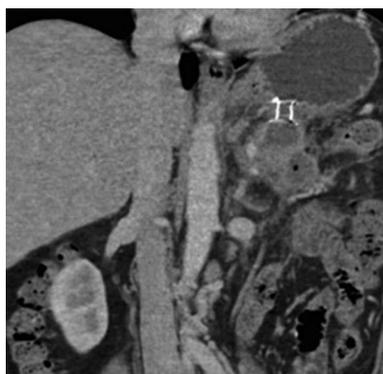
Figure 2 Computed tomography abdomen with visible metal stent between stomach and residual walled-off necrosis 6 wk after endoscopic ultrasonography guided insertion (initial cyst diameter 14 cm).

necrosis open to an internal diameter of more than 1 cm lumen to allow direct and repeated endoscopic access for endoscopic necrosectomy and extraction of necrotic tissue (Figure 2). The Axios stent (Xlumena™) and the Aix stent (Leufen™) are examples of such stents designed for EUS guided insertion, but also other companies are now producing similar stents. The covered stent produced by Hananro has extraflanges at the gastric end to stop migration into the cyst (Table 3).

The yo-yo-like design of the new SEMS such as the Axios™ stent results in a lumen apposing effect[21,22]. This can be adventageous in pancreatic fluid collections with indeterminate wall adherence.

Recent studies demonstrate a lower occlusion rate and the need for only one stent insertion due to the large diameter, the option for endoscopic access to the cavity as clear advantages of SEMS compared to conventional stents[23-26]. The migration risk remains. Some interventionalists place a double pigtail stent through the metal stent to prevent stent dislocation.

One step devices

Several new developments aim to simplify the EUS guided technique and to combine the steps of puncture, dilatation/enlargement of the ostium and drainage in one single tool.

The cystostome already combines the cyst puncture with an inner needle knife catheter followed by the dia-

thermy by a metal ring at the tip of the outer sheet[27]. Exchange over a wire for stenting is still necessary. However, a new development also now comes with a preloaded straight stent which can be placed directly after diathermy.

The Giovannini stent device[28] is an all-in-one stent introduction system combining a 0.035 needle-wire suitable for cutting diathermy, a 5.5 F guiding catheter and a preloaded straight plastic stent (8, 5 or 10 F, 5-cm-long). The needle-wire is introduced under EUS-guidance into the fluid collection using cutting current. After removing the internal rigid part, the wire can be curled in the cystic cavity to stabilize the position. The guiding and dilatation catheter follows over the wire and finally the straight plastic stent can be transmurally positioned (Giovannini Needle Wire Oasis; Cook endoscopy, Winston-Salem, NC, United States). This one-step EUS-guided technique for transmural cyst access has proven safe and effective for the management of pancreatic pseudocysts and abscesses[29].

A new combination tool is the "Naxix-access-device" (Xlumena™) which consists of a 19 G trocar with a short extandable side blade. The side blade enlarges the ostium to 3.5 mm diameter which allows to advance a pear shaped anquoring and a 10 mm dilatation balloon. The device has also additional channels for guide wires and contrast injection[30]. Avoiding device exchanges, this accessory allows access, guidewire insertion, tract enlargement and dilatation.

The recent technical developments have provided us with easier deployable stent systems for the EUS-guided management of pancreatic fluid collections. The SEMS appear safer and more effective in hitherto existing case series and studies. Large-sized, randomised comparative studies are required for further evaluation and this will lead to continued improvement of the techniques.

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Celiac plexus neurolysis in pancreatic cancer: The endoscopic ultrasound approach

Andrada Seicean

Andrada Seicean, Regional Institute of Gastroenterology and Hepatology Cluj-Napoca, University of Medicine and Pharmacy "Iuliu Hatieganu", 400162 Cluj-Napoca, Romania
Author contributions: Seicean A solely wrote this manuscript.
Correspondence to: Andrada Seicean, MD, PhD, Associate Professor, Regional Institute of Gastroenterology and Hepatology Cluj-Napoca, University of Medicine and Pharmacy "Iuliu Hatieganu", Croitorilor Street 19-21, 400162 Cluj-Napoca, Romania. andradaseicean@yahoo.com
Telephone: +40-74-4332107 Fax: +40-26-4431758

Abstract

Pain in pancreatic cancer is often a major problem of treatment. Administration of opioids is frequently limited by side effects or insufficient analgesia. Endoscopic ultrasound-guided celiac plexus neurolysis (EUS-CPN) represents an alternative for the palliative treatment of visceral pain in patients with pancreatic cancer. This review focuses on the indications, technique, outcomes of EUS-CPN and predictors of pain relief. EUS-CPN should be considered as the adjunct method to standard pain management. It moderately reduces pain in pancreatic cancer, without eliminating it. Nearly all patients need to continue opioid use, often at a constant dose. The effect on quality of life is controversial and survival is not influenced. The approach could be done in the central position of the celiac axis, which is easy to perform, or in the bilateral position of the celiac axis, with similar results in terms of pain alleviation. The EUS-CPN with multiple intraganglia injection approach seems to have better results, although extended studies are still needed. Further trials are required to enable more confident conclusions regarding timing, quantity of alcohol injected and the method of choice. Severe complications have rarely been reported, and great care should be taken in choosing the site of alcohol injection.

Key words: Endoscopic ultrasound; Celiac neurolysis; Pancreas; Cancer; Pain

Core tip: Endoscopic ultrasound-guided celiac plexus neurolysis should be considered as the adjunct method to standard pain management. It moderately reduces pain in pancreatic cancer, without eliminating it. Nearly all patients need to continue opioid use, often at a constant dose. The central technique is easy to perform, but intraganglia injection seems to give better results. This review focuses on methods of celiac neurolysis, with details about endoscopic ultrasound-guided celiac plexus neurolysis, indications and outcomes with regard to efficacy and safety, novel techniques, and predictors of pain response.

INTRODUCTION

The incidence of pancreatic cancer has increased over the last decade[1,2]. Few of the patients are diagnosed at a resectable stage (12%-20%)[3,4] and vascular resection during duodenopancreatectomy increases the 30-d postoperative morbidity and mortality rate[5]. For pancreatic cancer patients, the standardized net survival at 5 years is 6% for men and 10% for women[6]. Thus, palliative treatment is crucial in management. In this context, one of the most important symptoms to treat is pain. In the initial phase, the pain is visceral, but with disease progression, somatic pain may occur, especially due to the peripancreatic invasion of neural structures or muscles. Me-

dicinal palliation of pain from pancreatic cancer begins with non-opioid drugs, such as paracetamol, stepping up to opioids, such as tramadol, and, eventually, more powerful opioids, such as morphine or fentanyl. However, the dosage of opioid medication sometimes reaches a limit level due to side effects, such as nausea, constipation, somnolence, addiction, confusion or respiratory depression, and failure in achieving adequate analgesia. In these situations, neurodestructive methods involving the main pancreatic pain pathways, such as celiac block or thoracoscopic splanchnicectomy, seem efficient.

The celiac "plexus" is the largest plexus of the sympathetic nervous system, innervating the upper abdominal organs (pancreas, diaphragm, liver, spleen, adrenal glands, kidneys, abdominal aorta, mesentery, stomach, small bowel, ascending colon and the proximal portion of the transverse colon). The celiac plexus is situated within the retroperitoneal space posterior to the stomach and pancreas, close to the celiac axis, and it is separated from the vertebral column by the crush of the diaphragm. It comprises a dense network of ganglia around the aorta, with considerable variability in size (0.5-4.5 cm), number[7-11] and position (from the T12-L1 disc space to the middle of the L2 vertebral body). The left celiac plexus is typically located more caudally than its counterpart on the right. Celiac neurolysis may target either the plexus or the ganglia.

The preganglionic sympathetic fibres of the celiac plexus are grouped into the greater (T5-10), lesser (T10-11) and the least (T12) splanchnic nerves, and the plexus also receives parasympathetic fibres from the celiac branch of the right vagus nerve. All of these fibres are interrupted during thoracoscopic splanchnicectomy performed under general anaesthesia (Figure 1).

This review focuses on the following aspects: methods of celiac plexus neurolysis, with details about endoscopic ultrasound-guided celiac plexus neurolysis (EUS-CPN), indications and outcomes, including efficacy, predictors of pain response, safety and novel techniques.

CELIAC PLEXUS NEUROLYSIS

This involves chemical destruction of celiac ganglia and corresponding neural pathways by injecting dehydrated alcohol into the network of the celiac plexus. The result is moderate neuronal degeneration associated with residual fibrosis[7].

The initial method involved a posterior approach, accomplished under guidance by fluoroscopy or computed tomography (CT). The pain level at 12 wk after the procedure was significantly lower than with systemic analgesic therapy[8]. Unfortunately, in some cases the pleura or neural structures were accidentally touched by the needle, with subsequent development of serious side effects such as pneumothorax and paraplegia, respectively[8].

As a consequence, the anterior approach came to be considered a better option for CPN, accomplished either transcutaneously under ultrasound (US) guidance (developed in 1995[9]), CT guidance or endoscopic US (EUS)
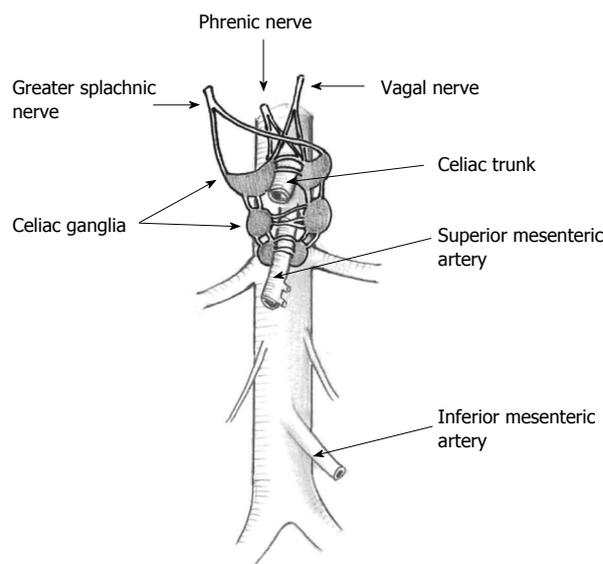
Figure 1 Anatomy of the celiac area (courtesy of Dr. Gombosiu C).

guidance (introduced in 1996[10]) or, more invasively, by means of surgery. More recently, the laparoscopic technique has been implemented[11,12].

The advantages of the EUS approach are the fine orientation of the needle above or lateral to the celiac trunk and the real-time performance of the procedure, under Doppler control of vessel interposition. In addition, the technique is easy, requiring only 2-3 min immediately after the staging or sampling of an inoperable pancreatic tumour. Better results can be expected owing to the better orientation of the needle, compared to the US or CT approach, and the real-time accomplishment of the procedure.

The technique consists of preprocedural hydration with 500 mL saline, followed by CPN performed with the patient in the left lateral position, under either general anaesthesia with propofol, or deep intravenous sedation with 2-4 mg of midazolam. Some endosonographers favour antibiotic prophylaxis, avoiding a retroperitoneal abscess[13-16], although alcohol is considered to be a bactericidal agent[17]. Bacterial translocation from the gut can be reduced by performing a single needle pass and by avoiding simultaneous gastric acid suppression treatment[17,18]. After colour Doppler assessment of vessel-gut interposition, a therapeutic linear-array echo-endoscope is used and the puncture site is chosen. Proximity to the diaphragm should be avoided, because of the potential for immediate pain due to the spread of alcohol. The devices used are 22-G or 19-G needles, or preferably fenestrated 20-G needles especially designed for EUS-CPN (Cook Medical, Winston-Salem, NC, United States). Recently, the use of a forward-viewing echo-endoscope has been reported in five patients[19].

For central injection, which is easier to perform, the needle is advanced above the celiac trunk, in the space between the aorta and the origin of the celiac axis. If bilateral injection is chosen, the echo-endoscope, situated

Figure 2 Endoscopic ultrasound images showing the position of the needle above the celiac plexus.

above the celiac axis, is rotated to one side until the origin of the celiac axis is no longer seen, and half of the entire solution is injected; the procedure is then repeated on the opposite side.

When ganglia are targeted, the echo-endoscope is rotated clockwise and celiac ganglia are found above the celiac trunk, alongside the trunk, and below the trunk, just above the superior mesenteric artery takeoff[20]. The ganglia are small hypoechoic nodules with hyperechoic foci in the center. Sometimes their interconnection can be seen. In large ganglia, thin linear hypoechoic lines arising from the edges of the ganglion are suggestive of small neural fibres[20]. The rate of ganglia detection varies between 79%-89% and it may also vary among endosonographers (65%-97%)[21-23]. As many ganglia as possible should be injected. The actual recommendations are to start the injection in the central part of the ganglia for those within 1 cm in diameter, or in the deepest part of the larger ganglia, and to perform the injection during the withdrawal of the needle, but only inside the ganglia[24].

Following the Doppler assessment of the area, aspiration is performed in order to rule out placement of the needle inside a vessel, which may lead to severe complications (Figure 2). Any lack of resistance during injection might suggest that the vascular space has been punctured; the needle should be withdrawn, and the aspiration test repeated. The injection starts with 3-10 mL of a local analgesic to prevent transient pain exacerbation induced by the neurolytic agent. Lidocaine 1%-2%[13,14,20,25], or a better analgesic such as bupivacaine 0.25%-0.75%[10,23,26-28], can be used. Subsequently, 10-20 mL of a neurolytic agent (98% dehydrated alcohol) is injected and a hyperechoic cloud is seen in the area of the needle tip as the substance spreads.

All patients must be kept under close observation for 2 h after the procedure, to monitor blood pressure, heart rate and temperature, and to identify any immediate complications.

Technical difficulties may occur in some cases because the anatomical landmarks could not be properly visualized. For example, during the bilateral technique, after

one side injection, the alcohol spreads and impedes the view of the opposite side. Sometimes, the celiac plexus region cannot be reached with the needle, as is the case in patients with cachexia who have very little fat tissue around the aorta, or when the diaphragm insertion is too close to the celiac trunk.

Other approaches such as thoracoscopic splanchnicectomy, EUS-guided direct celiac ganglion irradiation with ^{125}I seeds or radiofrequency ablation can be considered as alternative methods for celiac plexus destruction[11,29,30].

INDICATIONS OF EUS-CPN

The NCCN guidelines, version 1.2013 for pancreatic adenocarcinoma, recommend EUS-CPN for the treatment of severe tumour-associated pain. This technique is useful especially when intolerable adverse effects of opioid therapy occur, such as drowsiness, delirium, dry mouth, anorexia, constipation, nausea and vomiting, or an analgesic "ceiling" is seen due to neurotoxicity. In the case of jaundice caused by an unresectable pancreatic head tumour, biliary drainage should be offered first, followed by open CPN if pain persists[31].

Relative contraindications to EUS-CPN include difficult access due to anatomical distortion from previous surgery or congenital malformations. The absolute contraindications for EUS-CPN are the same as for any other invasive procedure: coagulopathy, platelets < 50000, and patients who are unable or unwilling to cooperate[32].

OUTCOMES

Efficacy for EUS-CPN

The main goals when performing EUS-CPN are the alleviation of pain and the improvement of quality of life. This procedure added no benefits regarding survival in two randomized controlled trials[13,27].

Although the quality of life was unchanged after CPN in one randomized trial[16], there are reports of improvement of parameters of quality of life such as functional status, work capability, sleep, and enjoyment of leisure activities[14,26]. The occurrence and duration of terminal delirium have also been reported as reduced after this procedure[33].

The assessment of pain intensity in chronic cancer patients uses different measurement scales. Visual analogue scales have proved to be less suitable in old patients with opioid use, due to limited communication skills and cognitive impairment during the last days of life, making self-reporting of pain more difficult[34]. Numerical rating scales are preferable in assessing cancer pain exacerbations to verbal rating scales[35]. As a result, observation of pain-related behaviours and discomfort is indicated in patients with cognitive impairment, in order to assess the presence of pain[36-38], and multidimensional questionnaires which evaluate the pain intensity together with other parameters of interference with pain are useful[26].

Although the real benefit of EUS-CPN compared

Table 1 Pain relief and techniques used in patients with endoscopic ultrasound-guided celiac plexus neurolysis

Ref.	n	Pain evaluation	Technique of EUS-CPN	Follow-up period (wk)	Pain alleviation
Doi et al[23] 2013	68	Numeric rating scale	Ganglia vs central	1	73% vs 45%
Leblanc et al[14] 2013	20	Numeric rating scale	Ganglia + central	6	90%
Seicean et al[26] 2013	32	Brief pain inventory	Central	2	75%
Wiechowska-Kozłowska et al[25] 2012	29	Numeric rating scale	Central + bilateral	8-12	76%
Wyse et al[27] 2011	48	Likert scale	Bilateral	12	60.70%
LeBlanc et al[13] 2011	50	Numeric rating scale	Central vs bilateral	14	69% vs 81%
Iwata et al[39] 2011	47	Visual analogue scale	Central	1	68.10%
Ascunce et al[20] 2011	64	Numeric rating scale	Ganglia or bilateral	1	50%
Sakamoto et al[40] 2010	67	Visual analogue scale	Under celiac trunk	4	33%-93%
Sahai et al[41] 2009	160	Visual analogue scale	Central vs bilateral	1	70% vs 45%
Ramirez-Luna et al[42] 2008	10	Visual analogue scale	Central	4	72.20%
Levy et al[15] 2008	17	General descriptors	Ganglia	4	94%
Tran et al[28] 2006	10	Numeric rating scale	Central	Not stated	70%
Gunaratnam et al[43] 2001	58	Visual analogue scale	Bilateral	24	78%
Wiersema et al[10] 1996	30	Visual analogue scale	Bilateral	12	79%-88%

EUS-CPN: Endoscopic ultrasound-guided celiac plexus neurolysis.

to placebo has not been studied, pain relief after the procedure varies between 45%-94% in different papers (Table 1). Two subsequent meta-analyses showed a mean rate of pain alleviation of 72%-80%, with a much lower rate of complete pain response[24,42,43]. However, many of the patients still required the same dose of analgesic and EUS-CPN should be considered as an adjunct method to standard pain management[24]. The post-neurolytic residual pain could be related to non-visceral pain, due to the invasion of the muscles or surrounding connective tissue, but factors concerning the technique used (type of technique, quantity of alcohol injected, timing of the procedure) have not been extensively studied.

The type of technique used for obtaining the best response is still controversial. Eleven studies on the central or bilateral technique have been published to date, showing a pain alleviation rate of 50%-88% at 1-14 wk after the procedure (Table 1). Bilateral technique, used in six of these studies, was associated with a rate of pain alleviation of 45%-88%, while central technique showed 68%-72% alleviation. To date, only one randomized controlled trial has compared the central and bilateral techniques of EUS-CPN and showed no difference in duration of pain relief (11 wk vs 14 wk), complete pain relief (2/29 patients vs 2/21 patients) or reduction in pain medication (9/29 patients vs 7/21 patients)[13]. The choice between the central or bilateral technique remains difficult, depending on the personal skills and the experience of every endosonographer. Our experience has showed good results with the central technique, which we consider easier to perform[26].

EUS-guided direct ganglia neurolysis, first reported by Levy in 2007, showed much better results in terms of pain alleviation (7 of 17 patients, 94%) at 2-4 wk; the known side effects - diarrhoea or hypotension - were noted. For the first time, long-lasting postprocedural pain relief (2.2 d) was reported in 7 of 17 patients[15]. One-week follow-up of pain alleviation showed better results for EUS-CPN at the celiac ganglia compared to EUS-CPN at the celiac trunk region using bilateral injection

(67.5% vs 33%)[14]. However, this technique has been used in only a few studies. One randomized controlled trial compared direct ganglia neurolysis with central neurolysis. The positive response rate at day 7 and the complete response rate were higher in the ganglia neurolysis group (75.5% vs 45.5% and 50% vs 18.2%, respectively)[23].

Early vs late injection
A randomized, double-blind, controlled trial in 96 patients showed that CPN was effective in pain reduction at 1-mo and 3-mo follow-up, but opioid consumption was constant - although it increased in the control group[27]. In the group of patients without radiochemotherapy, pain was significantly reduced and the need for increased opioids was prevented. In patients with radiochemotherapy, on the other hand, pain was significantly reduced only at 3 mo of follow-up. The authors concluded that this technique would be effective only for patients who refuse, or are ineligible for radiochemotherapy[27].

Amount of ethanol injection
The majority of cases have been performed using 10-20 mL alcohol[13-15,26,27]. Only one study compared the results when 10 or 20 mL alcohol injection was used during intraganglia or central injection and no difference in pain alleviation was noted[14].

Repetitiveness of the procedure
The benefit of repeated EUS-CPN was studied in 24 patients and results are less encouraging. The rate of successful pain relief was much lower than for the first EUS-CPN (29% vs 67% at 1-mo follow-up), and disease progression was a factor which limited the response[44].

EFFICACY FOR PERCUTANEOUS CPN AND ALTERNATIVE APPROACHES
Two important meta-analyses of the percutaneous approach have been published. The first one included 1117

Table 2 Immediate and late complications reported for endoscopic ultrasound-guided celiac plexus neurolysis in adenocarcinoma patients

Ref.	No. of procedures	Complications	Indication	Technique	Substance
Muscatiello et al[16]	1	Retroperitoneal abscess	PC	Not stated	Alcohol + bupivacaine
Gimeno-García et al[49]	1	Celiac axis infarction, kidney, splenic, hepatic infarction, death	PC	Bilateral	Alcohol + bupivacaine
Fujii et al[50]	1	Anterior spinal cord infarction with lower paraplegia	PC	Ganglia + central	Alcohol + bupivacaine
Wiechowska-Kozłowska et al[25]	29	Hypotension-1 Pain exacerbation-2 Transient diarrhoea-3	PC	Bilateral + central	Alcohol + bupivacaine
Mittal et al[53]	1	Anterior spinal cord infarct with lower paraplegia	PC	Ganglia + central	Alcohol + bupivacaine
O'Toole et al[17]	31	Hypotension-1	PC	Bilateral	Alcohol + bupivacaine
Levy et al[15]	17	Pain exacerbation-2	PC	Ganglia	Alcohol + bupivacaine
Leblanc et al[14]	20	Lightheadedness-1 Transient diarrhea-2 Transient nausea and vomiting-3	PC	Central + ganglia	Alcohol+ bupivacaine
Doi et al[23]	68	Transient hypotension-3 Upper gastrointestinal bleeding-1 Pain exacerbation-17 Transient diarrhea-5 Inebration-2	PC	Central + ganglia	Alcohol + bupivacaine
Jang et al[52]	1	Liver and splenic infarction, ischemia of the stomach and small bowel	Pancreatic metastasis	Central	Alcohol + bupivacaine + triamcinolone

PC: Plexus neurolysis in adenocarcinoma.

patients, 63% of them with pancreatic cancer, in whom bilateral X-ray-, US- or CT-guided neurolysis was performed. Pain alleviation at 2 wk was excellent; 90% relief was recorded at 3 mo after the procedure, and 70%-90% of patients experienced pain relief right up to the time of death. Transient pain was seen in most of the patients under study (96% in two of the studies analysed), transient diarrhoea in 44%, and transient hypotension in 38%. Severe neurological side effects were noted in 5 of the 268 patients (1%)[45]. A second meta-analysis, including 358 patients with CT-guided CPN, from six randomized controlled trials, showed a limited advantage in pain alleviation at 4 and 8 wk (0.42 and 0.44, respectively, on a visual analogue scale of 0-10), but opioid consumption was significantly lower, with fewer side effects[46].

To date, only one published randomized trial has compared the efficacy of CPN and thoracoscopic splanchnicectomy, and the results were not significant compared to the control medical management group. The main limitation of the study was the small number of patients included in each arm of the study[11].

EUS-guided direct celiac ganglion irradiation with ^{125}I seeds was performed in 23 patients, with significant pain reduction 2 wk later. Initial pain exacerbation was seen in 26% of the patients, but no major complications occurred up to the time of death[29].

Radiofrequency ablations of pancreatic mass and celiac plexus have been reported as successful in the treatment of chronic pain[30].

Predictors of response rate

A retrospective study compared the results of ganglia injection with those of non-direct ganglia injection (40

vs 24 patients). The median number of visualized ganglia was two. The pain response rate was 50% at 1 wk, 77% at 30 d, and opioid consumption was 57% lower at 1-wk follow-up. Pain alleviation was significantly lower for patients in whom the ganglia were not visualized, and it was also lower, albeit not significantly so, for tumours located in the body or tail of the pancreas, for large tumours and for patients with severe pain at presentation[20].

A second study of 47 patients with central-injection CPN showed 68% pain alleviation at 1 wk. The predictors of poor pain alleviation were direct invasion of celiac ganglia and left diffusion of the neurolytic agent[37].

Safety

Many complications have been described for EUS celiac block indicated for chronic pancreatitis, and some of these complications have been seen in EUS-CPN for pancreatic cancer, such as transient diarrhoea (4%-15%) and transient hypotension (1%)[14,18,47,48] or alcohol intolerance. Nowadays, it is considered that the potential immediate complications are rare, such as hypotension, tachycardia, initial pain enhancement, severe bleeding and paraplegia. The late side effects include diarrhoea, hypotension, fever and paraplegia[48].

Recently, severe complications of EUS-CPN have been reported in individual cases, and endosonographers should be aware of them (Table 2). Permanent lower paraplegia, due to spinal cord infarction, was noted in one patient; the mechanism was considered to be alcohol diffusion *via* the left T12 intercostal artery towards the anterior spinal artery, or vasospasm caused by alcohol or acute thrombosis due to injection of a high volume into the celiac area[50-53]. Injury of the lumbar artery leading

into the artery of Adamkiewicz could be involved. This major artery originates from the aorta, varies in position from T7 to L4, supplies the lower two-thirds of the anterior spinal artery, and it is anatomically closely related to the celiac ganglion[53]. The clinical manifestations, reported 14 h after the procedure, comprised motor weakness, decreased pain and temperature sensation below T7-L1, and detrusor atony. Prolonged periprocedural hypotension may have played a part[53]. Extreme caution should be taken concerning the placement of the needle tip, including Doppler US examination of the area and aspiration before injection.

Another complication was thrombosis of the celiac trunk, with wall thickening and bubble-like pneumatosis of the stomach, duodenum, jejunum, ileal loops and ascending colon. Signs of hepatic infarction of segment I and III, near-total right kidney, as well as splenic infarction were discovered, and the evolution was fatal. The explanation was the sclerosing effect of alcohol after injection[49]. Alcohol neurolysis for treatment of chronic pancreatitis has been recorded as leading to necrosis and perforation of the stomach and aorta with lethal outcome[51], as well as splenic, gastric and pancreatic infarction[52]. The infarction of the liver, spleen, stomach, and proximal small bowel after celiac neurolysis for pancreatic metastasis was reported in one case, as vasospasm resulted from the diffusion of ethanol into the celiac artery[54].

Retroperitoneal abscesses have been previously noted in chronic pancreatitis patients with triamcinolone injection, especially after gastric acid supression therapy[17,18], but one case has been described in pancreatic cancer, too[16]. However, antibiotic prophylaxis has not been carried out in many studies[23,25-28].

Initial pain exacerbation after EUS-CPN was reported in up to 29%-34% of cases[15,22]. Previous studies considered initial pain exacerbation as a sign of greater pain relief at follow-up[15], but this was not confirmed in further studies[22].

Novel techniques

In the attempt to improve the technique, Sakamoto et al[40] used broad plexus neurolysis near the superior mesenteric artery with the aim of administering the neurolytic agent to a larger number of ganglia. The authors checked the spread of the neurolytic agent around the celiac axis and showed that the new technique achieved neurolysis in five or six areas in a higher proportion of patients than the previous method. With regard to the pain levels at 7 d and 30 d, significant reduction was obtained for five and six areas of neurolytic agent diffusion, but not for three or four areas. However, this study had some limitations: there were methodological problems, the physicians' experience increased during the study, not all patients (only 60 of 67) had pancreatic cancer, and the overall success rate for pain alleviation was only 50% at 30 d, the lowest rate ever reported at that time[38].

CONCLUSION

EUS-CPN should be considered as an adjunct method to standard pain management. It moderately eases pain in pancreatic cancer without eliminating it completely. Nearly all patients need to continue opioid use, often at a constant dose. Multicentre, randomized, controlled trials are required to provide more reliable conclusions on timing, quantity of alcohol injected and the method of choice. Until then, in the light of the severe complications reported recently, great care should be taken when choosing the site of alcohol injection.

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2014 ADVANCES IN PANCREATIC CANCER

Celiac plexus neurolysis in the management of unresectable pancreatic cancer: When and how?

Jonathan M Wyse, Yen-I Chen, Anand V Sahai

Jonathan M Wyse, Division of Gastroenterology, Jewish General Hospital, McGill University, Montreal H3T 1E2, Quebec, Canada

Yen-I Chen, McGill University Health Center, McGill University, Montreal H3A 1A1, Quebec, Canada

Anand V Sahai, Division of Gastroenterology, Hopital Saint Luc, Centre Hospitaliér de l'Universite de Montréal, Montreal H2X 1P1, Quebec, Canada

Author contributions: All author contributed equally to the preparation of this manuscript.

Correspondence to: Jonathan M Wyse, MD, MSc, Division of Gastroenterology, Jewish General Hospital, McGill University, 3755 Chemin de la Cote-Ste-Catherine, Montreal H3T 1E2, Quebec, Canada. jonathan.wyse@mcgill.ca

Telephone: +1-514-3408286 Fax: +1-514-3408282

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Core tip: The efficacy of salvage celiac plexus neurolysis (CPN) either by percutaneous or endoscopic ultrasound (EUS) guided technique has been modest in its ability to reduce pain and narcotic requirements in patients with unresectable pancreatic cancer, and few studies with rigorous methodology exist. Data for early EUS-CPN at time of diagnosis appears to prevent pain escalation while moderating narcotic use and future studies should explore CPN for patients before rescue therapy is needed. Reports of serious and fatal complications of CPN have surfaced in recent years.

Abstract

Pancreatic cancer is the second most common abdominal cancer in North America with an estimated 20% resectability at diagnosis, and overall 5-year survival of 5%. Pain is common in pancreatic cancer patients with 70%-80% suffering substantial pain. Celiac plexus neurolysis (CPN) is a technique that can potentially improve pain control in pancreatic cancer while preventing further escalation of opioid consumption. CPN is performed by injecting absolute alcohol into the celiac plexus neural network of ganglia. This review sets out to explore the current status of CPN in non-resectable pancreatic cancer. We will examine: (1) the efficacy and safety of percutaneous-CPN and endoscopic ultrasound guided-CPN; (2) specific technique modifications including bilateral (*vs* central) injections and celiac ganglia neurolysis; and (3) the issue of CPN timing, early at pancreatic cancer diagnosis *vs* traditional late use as salvage therapy.

INTRODUCTION

Pancreatic cancer is the second most common abdominal cancer in North America with an estimated number of 45220 new diagnoses and 38460 deaths in the United States in 2013[1]. The high mortality rate is due in part to the aggressive nature of the tumor and its asymptomatic disease progression leading to delayed diagnosis with an estimated 20% resectability at diagnosis, and overall 5-year survival of 5%[2,3]. Pain is common in pancreatic cancer patients with 70%-80% suffering substantial pain[4-6]. As a result, systemic analgesic therapy (SAT)

usually including opioid medication is central to the management of unresectable pancreatic cancer. However, pain can often become intractable and refractory to narcotics leading to dose escalation and opioid associated side effects[7-9].

Celiac plexus neurolysis (CPN) is a technique that can potentially improve pain control in pancreatic cancer while preventing further escalation of opioid consumption[6,10]. CPN is most often performed by injecting local anesthetic followed by absolute alcohol into the celiac plexus neural network of ganglia with intention to ablate the tissue transmitting pain from the pancreas and adjacent visceral organs. In current clinical practice, it has been used almost exclusively as salvage therapy when pain control is inadequate with SAT[11]. CPN modalities include surgical splanchnectomy, percutaneous (PQ)-CPN, and endoscopic ultrasound guided (EUS)-CPN. Surgical splanchnectomy/intra-operative celiac plexus neurolysis can be performed on those not deemed inoperable preoperatively but will not be reviewed in this paper. The two most commonly practiced routes are the posterior PQ-CPN usually under CT or fluoroscopic guidance and EUS-CPN. There has been much controversy as to which route and which specific techniques should be the gold standard based on efficacy and safety. This is partially due to a lack of well-designed randomized controlled trials and lack of studies directly comparing the two modalities. Furthermore, there is recent data to suggest that using CPN as salvage therapy may not be the only or best option and that early CPN, performed at the time of diagnosis, may prevent or slow the spiral of increasing pain and opioid consumption[12].

This review sets out to explore the current status of CPN in non-resectable pancreatic cancer. We will examine: (1) the efficacy and safety of PQ-CPN and EUS-CPN; (2) specific technique modifications including bilateral (*vs* central) injections and celiac ganglia neurolysis (CGN); and (3) the issue of CPN timing; early at pancreatic cancer diagnosis *vs* traditional late use as salvage therapy.

PQ-CPN

Pain control

Initial meta-analyses regarding the use of PQ-CPN in controlling pain due pancreatic cancer showed conflicting results and are limited to mostly retrospective and uncontrolled studies[10,13,14]. Since then, several RCTs have been published of which 5 (265 patients) from 1993-2008 were analyzed in a recent systematic review[5,15-19]. They demonstrated statistically significant improved pain level in the PQ-CPN group compared to SAT at 1-2 wk by -0.87 [95%CI: -1.47-(-0.28), *P* = 0.004], and at 4 wk by -0.47 [95%CI: -0.71-(-0.23), *P* = 0.0001]. At 8 wk however, the statistical difference was lost -0.31 (95%CI: -0.74-0.12) and similarly no study showed benefit at 12 wk[18]. A previous meta-analysis, by Yan *et al*[6], also comprised of 5 RCTs (302 patients, 3 studies over-

lap with Nagels *et al*[18]) including one intra-operative neurolysis[5,6,16,17,20,21]. This analysis found pain improvement at 2, 4 and 8 wk of -0.34 (95%CI: -1.03-0.34, *P* = 0.33), -0.50 [95%CI: -0.85-(-0.15), *P* = 0.005], and -0.60 [95%CI: -0.82-(-0.37), *P* < 0.00001] respectively[6].

Regardless of the statistical significance found at different time points between these often heterogeneous studies within 2 meta-analyses, it is striking that all of the point estimates are less than one. A decrease of less than one point on a pain scale is unlikely to be clinically significant and questions whether the procedure is beneficial at all. The difficulty in interpreting the true clinical significance lies in the fact that opioid consumption (see below) is a direct confounder of pain and both pain and opioid use are routinely analyzed with univariate statistical models. If opioid consumption were to simultaneously decrease or even remain unchanged relative to the SAT groups then the difference in pain corrected for opioid use may become clinically significant (data unavailable).

Opioid consumption

To allow for some comparison, data from the 2 above meta-analyses will be used. Nagels *et al*[18] found an absolute reduction in opioid use compared to SAT at 2 wk of -44.64 mg [95%CI: -72.74-(-16.54), *P* = 0.002], 4 wk -72.41 mg [95%CI: -86.14-(-58.68), *P* < 0.00001], 8 wk -70.02 mg [95%CI: -104.05-(-36.00), *P* < 0.0001] and one study at 12 wk (105 ± 65 mg *vs* 169 ± 71 mg, *P* < 0.01)[18]. Yan *et al*[6] found similar findings of decreased opioid use with PQ-CPN at 2 wk -39.99 mg [95%CI: -60.08-(-19.91), *P* < 0.0001], 4 wk -53.69 mg [95%CI: -79.65-(-27.73), *P* < 0.0001] and 8 wk -80.45 mg [95%CI: -134.66-(-26.24), *P* = 0.004].

Some of the above differences in opioid requirements do seem clinically significant, but as mentioned, to measure their true benefit a bivariate or multi-variate analysis would be necessary. These studies also did not convincingly show a decrease benefit in opioid related side effects. However, as discussed below this patient population has symptoms impacted by numerous factors including multiple medications, psycho-social stressors, and mobility. Therefore, to isolate constipation (for example) as strictly a narcotic induced side-effect is likely inappropriate.

Quality of life

Finally, when assessing the effect of PQ-CPN on quality of life (QOL), the data is inconclusive with some studies suggesting an improvement while others failing to demonstrate a significant difference[5,6,15,16,18,19].

It is important to note that the patient population being dealt with are palliative patients at the end of their life. Pain is an extremely complex entity at baseline, and its complexity is only enhanced in patients with a growing and spreading tumor who are facing their own mortality. Although the overall impact on QOL remains controversial, a modest pain reduction in the context of

clinically significant opioid reduction may still be very meaningful. Furthermore, the QOL scales used varied widely and could not be easily combined in any meta-analysis, and the QOL categories themselves within these scales would not be expected to improve by better pain control alone. A simple question such as "did this procedure improve your life in a meaningful way?" may have more appropriately assessed its worthiness. Nevertheless, these concepts and issues still bring into question whether PQ-CPN as a last resort in salvage therapy should be recommended to these patients.

EUS-CPN

EUS-CPN has emerged as a promising approach to CPN that has the potential for better visualization of the celiac plexus through close proximity and real-time high-resolution ultrasound, possibly allowing for more precise and safer injections. However, the data supporting this approach once again in the context of salvage therapy are limited to uncontrolled retrospective studies. Wiersema and Wiersema[22] were the first to describe EUS-CPN in 58 patients and showed modest improvement in pain control up to 12 wk following therapy. More specifically, 45 patients (78%) experienced a decrease in pain score independently of narcotic use. Since then, there have been several other observational studies (with no control group) examining EUS-CPN in relieving pain due to pancreatic cancer[23-26]. In a systematic review of these studies, a significant pain reduction was noted at weeks 2, 4, 8, and 12 with a mean difference in pain score of -4.26 [95%CI: -5.53-(-3.00)], -4.21 [95%CI: -5.29-(-3.13)], -4.13 [95%CI: -4.84-(-3.43)], -4.28 [95%CI: -5.63-(-2.94)] respectively[18]. This is consistent with a meta-analysis, which showed a pain reduction in 80% of the patients following EUS-CPN for pancreatic cancer[27]. EUS-CPN studies showed relatively stable or slightly lower opioid requirements that paralleled this pain reduction[16,17,19]; however, there is no randomized controlled study for EUS-CPN used specifically as salvage therapy despite these promising data.

ADVERSE EVENTS ASSOCIATED WITH CPN

PQ-CPN

It is important to distinguish common or even expected side effects from CPN complications. Frequent minor adverse events associated with PQ-CPN are believed to be due to disturbances of the autonomic system resulting from ablation of the celiac plexus and sympathetic blockade leading to unopposed parasympathetic activity. One study estimated diarrhea (9%), hypotension (8%), constipation (40%), nausea and vomiting (41%), and lethargy (49%)[6]. Pain at the site of injection (96%) has also been frequently reported[10]. Rare complications are described in case reports and include lower neurological deficit (weakness and paresthesia), pneumothorax, and

hematuria; and are estimated to occur at 2%[10]. Paraplegia itself is believed to occur secondary to needle trauma or vasospasm induced by the injection of alcohol into the artery of Adamkiewicz leading to ischemic cord injury *via* the anterior spinal artery. Paraplegia has been reported in the literature and is estimated to occur in less than 0.15% of the cases[28].

EUS-CPN

Data on adverse events of EUS-CPN are limited to small retrospective studies and case reports. Similar minor peri-procedural events such as transient hypotension were described in 3 case series and estimated at 11%[23,25,26]. Diarrhea was noted in 4 studies in approximately 18%[23,24,26,29]. Transient abdominal pain was described in case series at rates varying from 1.5% to 8%[22,23,25,26]. Theoretically, EUS might be the safer modality. Its anterior approach through the gastric wall allows for direct passage of the needle into the target area while visualizing and avoiding vascular structures, without having to traverse the retrocrural space near other vital organs. Although initial reports (prior to 2012) of serious adverse events were lacking, there have been a number of severe complications recently reported in the literature. Gimeno-Garcia *et al*[30] reported the first fatal complication with EUS-CPN in the context of chronic pancreatitis leading to celiac artery thrombosis and vasospasm resulting in multi-organ ischemic injury and death. Subsequently, 2 additional reports of ischemic injury and death following EUS-CPN, were also believed to be due to injection of ethanol into the celiac artery leading to vasospasm[31,32]. Other reported complications include retroperitoneal bleeding, and 2 cases of paraplegia[30,33,34].

Overall, although EUS may potentially enhance precision of injections, no conclusions can be made regarding the safer modality without head to head studies with PQ-CPN. Furthermore, serious fatal complications although rare are not unavoidable with EUS-guided therapy.

PQ-CPN *VS* EUS-GUIDED CPN

There are no studies directly comparing EUS-CPN and PQ-CPN in the management of pancreatic cancer. Efficacy of celiac plexus block (CPB) for chronic pancreatitis pain (using an anesthetic agent ± steroids as opposed to ethanol in neurolysis for pancreas cancer) remains controversial. However, two RCTs comparing EUS and PQ-CPN in CPB for chronic pancreatitis have suggested greater efficacy with EUS-CPB than PQ-CPB[35,36]. Gress *et al*[35] studied 20 patients showing greater and more persistent pain relief up to 12 wk post-treatment favoring EUS-CPB. Major weaknesses in this study; however, include its small sample size and unblinded methodology. Santosh *et al*[36] performed a larger, single-blinded RCT involving 56 patients favoring EUS-CPB over PQ-CPB for initial pain relief with 70% *vs* 30% responding to treatment respectively. Pain relief was also shown to be

more persistent with 38% *vs* 10% having significant pain relief at 12 wk. Although data from these RCTs of CPB in chronic pancreatitis are not directly applicable to CPN in pancreatic cancer, they do suggest a potential superior efficacy with they do suggest a potential superior efficacy with EUS in terms of drug delivery. Trials comparing PQ and EUS-CPN are certainly needed.

BILATERAL OR UNILATERAL CPN AND CGN

Bilateral *vs* unilateral/central neurolysis

Unilateral neurolysis is accomplished by a single injection into the base of the celiac artery takeoff. This technique may not adequately expose celiac ganglia to ethanol as it is now appreciated that the majority of ganglia are between the celiac artery and left adrenal gland[37].

Bilateral injection, is performed by injecting into both sides of the celiac plexus by torqueing the echoendoscope to each side of the celiac artery and advancing the injection needle parallel to its trajectory. Although there is potential for more adverse events due to greater needle movement, the bilateral approach has been shown by some to be more effective in providing pain relief. In a prospective cohort study comparing unilateral *vs* bilateral CPN or CPB, the bilateral technique achieved significantly more pain relief *vs* unilateral (mean percent pain reduction) 70.4% (61.0, 80.0) *vs* 45.9% (32.7, 57.4), $P = 0.0016$, at day 7 post treatment. Although this is a short-term study the onset of neurolysis effect begins soon after the nerve ablation, therefore a comparison between two techniques at 7 d can still be revealing. The only predictor of a > 50% pain reduction was bilateral injection [odds ratio 3.55, (95%CI: 1.72-7.34)][38]. Furthermore a meta-analysis also suggested superiority of pain reduction with bilateral injection over central injection, 85.54% *vs* 45.99% respectively[27]. A subsequent RCT comparing the two approaches in pancreatic cancer in 50 patients did not suggest a significant difference in terms of pain control or adverse events. However, there was a trend to nearly a 30% increase in duration of effect (11 wk *vs* 14 wk) in favor of bilateral, a result, which may have been limited by sample size[39]. Furthermore, the point estimate for central/unilateral seemed high at 69% (compared to approximately 45% in other studies) and may have prevented a difference from being detected. One must keep in mind that meta-analyses of the highest quality studies for both PQ-CPN and EUS-CPN included almost only the bilateral technique[6,27]. The bilateral technique also requires significant advancement on either side of the celiac artery and may be operator dependent. Finally, two other studies also support the notion that injection deeper[40] and along both sides of the celiac axis provide better pain relief[41]. Although there is no definitively proven superior technique, we favor the bilateral technique given the sum of the above evidence as well as the concept of wider distribution of the ethanol near areas where ganglia are most commonly found.

Central ganglia neurolysis

Recent developments in EUS equipment have improved resolution such that injection directly into celiac ganglia is possible in certain patients. One prospective study in 200 patients undergoing diagnostic EUS demonstrated a rate of celiac ganglia detection of 81%, Figure 1[42]. Another study demonstrated a ganglia visualization rate of 89% in 57 patients[43]. These percentages seem high in our experience nevertheless exemplify the real possibility of visualizing ganglia. Since ganglia are collections of nerve bodies and glial cells, injections into these structures have the potential to obliterate more neurons successfully, possibly leading to greater pain suppression. Levy *et al*[44] provided preliminary data on EUS-CGN demonstrating its safety and effectiveness in achieving significant pain relief in 94% of the subjects with pancreatic cancer. In addition, a retrospective analysis of EUS-CPN and CGN found that visualization of celiac ganglia was the best predictor of response to therapy[26]. Recently, an RCT comparing EUS-CGN *vs* EUS unilateral CPN showed substantial greater pain relief in the CGN group (73.5% *vs* 45.5%, $P = 0.026$) with similar adverse events[37]. However, the comparison was not against bilateral injection and the response rate with CGN *vs* unilateral was remarkably similar to the bilateral *vs* unilateral technique referenced above 70.4% (61.0, 80.0) *vs* 45.9% (32.7, 57.4), $P = 0.0016$[38].

Overall, superior efficacy of EUS-CGN is possible but unproven, especially compared to bilateral injection. This also lends biologic plausibility to bilateral injection being more efficacious than central since the ganglia frequently "and remove" as very are located lateral to the celiac artery and may be injected with the bilateral technique even when not visualized. CGN is also not possible *via* PQ-CPN. With EUS-guided CGN, although the drug is injected into the ganglia, it is conceivable that drug also diffuses beyond the targeted ganglia and destroys adjacent, invisible ganglia. Also, variation in equipment, make and model significantly impact ability to visualize ganglia and so success rates cannot be generalized. At this time we do not recommend CGN as a standard for CPN technique as it does not provide a wider distribution of the ethanol over the bilateral technique, but does add a degree of technical complexity and dependence on quality of equipment.

TIMING OF CPN: EARLY (NEAR TIME OF DIAGNOSIS) *VS* TRADITIONAL SALVAGE THERAPY

We hypothesize that one of the primary reasons why the magnitude of effect shown for salvage CPN is often seen as marginal and not clearly clinically meaningful is that it is offered "too late". Once pancreatic cancer has progressed causing increasing pain and tolerance to narcotics, a true rescue is unlikely to occur. The postulated advantage of early therapy therefore is to prevent
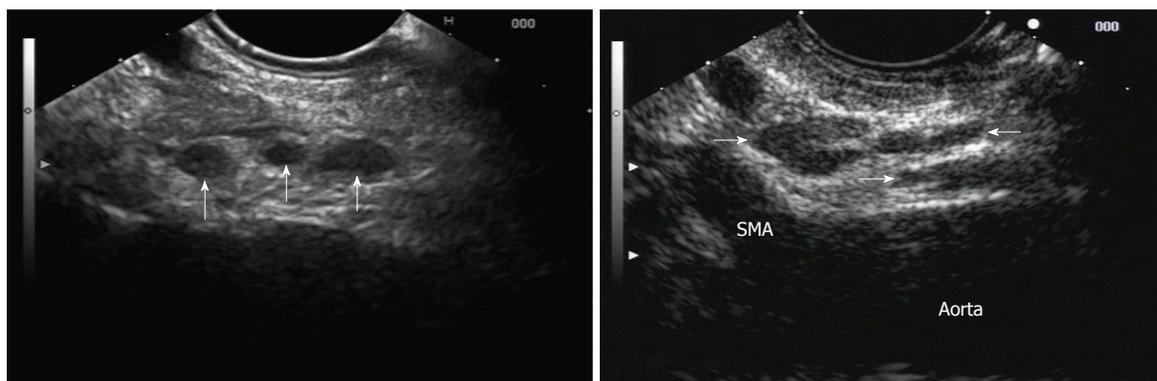
Figure 1 Three celiac ganglia are demonstrated in each image (arrows). SMA: Superior mesenteric artery.

or minimize both pain progression and narcotic dose escalation and tolerance. We addressed this issue in the first sham controlled RCT comparing early EUS-CPN (at the time of diagnosis) for unresectable pancreatic cancer *vs* standard SAT[12]. The difference in absolute mean change in pain between the early and salvage therapies were -1.0 [95%CI: -1.7-(-0.1)] at 1 mo and -2.2 [95%CI: -3.1-(-1.4)] at 3 mo favoring the early CPN group. Despite starting with a lower pain level than salvage therapy trials the absolute decrease in pain was greater than those found in the PQ-CPN RCTs above, and statistically significant at 3 mo. For difference in mean percent change in pain score the EUS-CPN group trended at 1 mo and was significantly greater at 3 mo as well, -28.9% (95%CI: -67.0-2.8, $P = 0.09$), and -60.7% [95%CI: -86.6-(-25.5), $P = 0.01$] respectively. In the SAT group, morphine use increased compared with baseline at both 1 mo (mean absolute change in MEQ consumption +54 mg [95%CI: +20-(+96)] and particularly at 3 mo (mean absolute change in MEQ consumption +100 mg [95%CI: +49-(+180)]. In the EUS-CPN group, morphine use also increased at 1 mo (mean change in MEQ consumption +53 mg [95%CI: +28-(+89)], but plateaued by 3 mo (mean change in MEQ consumption +50 mg [95%CI: +28-(+79)]. Comparing groups, EUS-CPN did not significantly reduce narcotic use at 1 mo, however, at 3 mo post-procedure there was a strong trend towards lower opioid consumption in the CPN group -49.5 mg (95%CI: -127.5-7.0, $P = 0.10$). Importantly, patients who did not receive subsequent radiation or chemotherapy demonstrated greater difference between groups. For example, a significant reduction in narcotic consumption was noted at 3 mo -144.5 mg [95%CI: -290.0-(-30.0)] (Table 1). The stronger results in patients who did not undergo adjuvant therapy underlies that this therapy with its inherent benefit to the patient, diluted the magnitude of effect of CPN alone. Therefore, data from this RCT suggest that early EUS-CPN prevents pain escalation while moderating narcotic use. Compared to all of the studies using salvage therapy, both PQ and EUS-CPN, these results seem very favorable.

CONCLUSION

Severe and intractable pain refractory to traditional SAT is a common occurrence of non-resectable pancreatic cancer. Pain control is crucial in the management of this population with several retrospective studies and a handful of RCTs demonstrating greater pain relief with equal and/or decreased opioid requirements with CPN (PQ or EUS). Although there are no head to head trials comparing EUS to PQ-CPN, data comparing the two modalities for CPB in chronic pancreatitis suggests EUS may be superior. Despite no conclusive data suggesting superiority, EUS does offer the potential for enhanced visualization of important vital structures and of celiac ganglia should CGN studies become more robust. Given the sum of the evidence and with wider distribution of ethanol in areas where ganglia are known to reside, we favor bilateral CPN over central injection. However this superiority is still controversial and central injections are certainly acceptable if the echoendoscopist is more comfortable with the latter. CGN cannot yet be recommended given inconsistent visualization of ganglia and the lack of trials compared to the bilateral technique which itself can be reproduced consistently in patients using only the celiac artery as a landmark. Perhaps most importantly, we feel there should be an emphasis of future studies on performing CPN early (at or near diagnosis) and the only existing EUS-CPN RCT did examine this approach with results comparable and seemingly superior to existing PQ-CPN RCTs done exclusively for salvage therapy. Preventing the escalation of pain and narcotic use should be the purpose of CPN in patients with unresectable pancreatic cancer. One must note, however, that pain due to pancreatic cancer is multi-factorial not only including celiac plexus pathways but also from, for example, intestinal obstruction and liver capsule distention from metastases. CPN will only target some of these pain mechanisms and may play less of a role as disease progresses and other pain etiologies become more pronounced.

Given the totality of existing evidence, it appears that in 2013, the optimal patient for successful and meaningful CPN would be undergoing diagnostic and staging

Table 1 Early endoscopic ultrasound-celiac plexus neurolysis *vs* systemic analgesic therapy: Pain relief and narcotic consumption with or without chemo-XRT[12]

EUS-CPN *vs* control	After 1 mo (95%CI)	After 3 mo (95%CI)
Difference in mean % change in pain relief:		
No chemo-XRT[1]	-59.6% (-95.4 to -27.6)[1]	-85.8% (-127.6 to -51.3)[1]
Chemo-XRT	31.0% (-34.3 to 106.2)	-45.6% (-72.6 to -23.3)[1]
Difference in mean change in MEQ consumption:		
No chemo-XRT	-2.4 mg (-58.4 to 60.8)	-144.5 mg (-291 to -30)[1]
Chemo-XRT	11.4 mg (-23.7 to 39.4)	26.1 mg (-12.2 to 56.5)

[1]Statistically significant $P < 0.05$. EUS-CPN: Endoscopic ultrasound-celiac plexus neurolysis; Chemo-XRT: Chemotherapy and radiation therapy; MEQ: Morphine equivalent.

EUS for pancreas cancer. CPN in this instance may be more impactful if the patient happens to not undergo subsequent chemotherapy/radiotherapy. Rare, serious, and even life threatening complications regardless of timing and route have to be disclosed and discussed with the patient in detail. Future studies should focus on early CPN in this unfortunate patient population.

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2014 ADVANCES IN PANCREATIC CANCER

Opioid growth factor and the treatment of human pancreatic cancer: A review

Ian S Zagon, Patricia J McLaughlin

Ian S Zagon, Patricia J McLaughlin, Department of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, Hershey, PA 17033, United States
Author contributions: Zagon IS and McLaughlin PJ contributed equally to this review.

Correspondence to: Patricia J McLaughlin, Professor, Department of Neural and Behavioral Sciences, Pennsylvania State University College of Medicine, MC-H109, 500 University Drive, Hershey, PA 17033, United States. pxm9@psu.edu
Telephone: +1-717-5316414 Fax: +1-717-5315003

Abstract

Opioid growth factor (OGF), chemically termed [Met^5]-enkephalin, and its receptor, OGF receptor (OGFr), form a biological axis that tonically regulates cell proliferation by delaying the G_1/S interface of the cell cycle under homeostatic conditions or in neoplasia. Modulation of the OGF-OGFr pathway mediates the course of pancreatic cancer, with exogenous OGF or upregulation of OGFr repressing growth of human pancreatic cancer cells in culture and in nude mice. OGF therapy alone or in combination with standard chemotherapies such as gemcitabine and 5-fluorouracil results in enhanced inhibition of DNA synthesis and tumor growth. Molecular manipulation of OGFr confirms that the receptor is specific for OGF's inhibitory action. Preclinical studies have warranted Phase I and Phase II clinical trials using OGF infusions as a treatment for patients with advanced, unresectable pancreatic cancers. OGF, an endogenous neuropeptide, is a safe, non-toxic, and effective biotherapy that utilizes the OGF-OGFr axis to mediate pancreatic tumor progression.

Core tip: Opioid growth factor (OGF) biotherapy for human pancreatic cancer is based on inhibition of DNA synthesis by upregulation of cyclin-dependent inhibitory kinases. Preclinical studies using human pancreatic cancer cell lines have demonstrated that OGF interaction with its selective receptor OGF receptor (OGFr) is a physiological determinant of cell proliferation. Addition of OGF to standard chemotherapies enhances the efficacy of treatment. Studies in nude mice confirm that the OGF-OGFr axis regulates pancreatic cancer progression. Clinical trials using OGF for treatment of patients with unresectable pancreatic tumors reveal that OGF is a novel endogenous opioid that is safe, non-toxic, elicits negligible side effects and reduces pancreatic tumor size in persons who have failed other therapies.

INTRODUCTION

Novel therapies for human pancreatic cancer are needed to treat the more than 45000 people in the United States who will be diagnosed with this cancer in 2013[1]. Death due to this cancer approaches the same number, with estimates of more than 38000 persons dying of pancreatic cancer in 2013. Males have increased incidence and

death rates relative to females; black ethnic groups have the highest incidence[1,2]. Five-year survival rates range between 5% and 6%, and have not changed in more than a decade of research[2]. Therapeutics based on underlying mechanisms of disease are needed.

The standard of care for pancreatic cancer is gemcitabine. This anti-metabolite is a nucleoside analog that blocks DNA replication, or inhibits ribonucleotide reductase, an enzyme needed to produce the deoxyribonucleotides necessary for DNA replication; both pathways induce apoptosis and slow tumor growth[3]. However, gemcitabine cannot be used if the patient has allergies (*e.g.*, dye, additives, food), other diseases (*e.g.*, kidney, liver, hepatitis, heart, lung, diabetes, gout), or infections. Radiation therapy cannot be combined with gemcitabine, and women of child-bearing age are encouraged not to take gemcitabine as it may cause birth defects[3].

New endogenous peptide pathways have been identified that provide novel targets for non-toxic therapeutic alternatives for pancreatic cancer. Our knowledge about the biology of pancreatic cancer supports the need for treatments that target the biology of this cancer.

ENDOGENOUS OPIOIDS

An endogenous opioid peptide and its receptor were first identified more than 3 decades ago as being an important inhibitor of human cancer cell proliferation[4,5]. Ongoing studies on the opioid growth factor (OGF)-OGF receptor (OGFr) axis have characterized this pathway and defined mechanistic approaches for the treatment of neoplasia[6]. The peptide is chemically termed [Met^5]-enkephalin, and is a 5-amino acid neuropeptide secreted by the brain and originally identified as an endogenous opioid by scientists in the mid-1970s[7-9]. This peptide was renamed OGF after discoveries of its growth modulating characteristics in mouse neuroblastoma cells and developing rat brain[4,5,10], and to distinguish its pharmacological function from neurotransmission. OGF is derived from both preproenkephalin and pro-opiomelanocortin genes[11], and is rapidly translated and degraded in human blood. Studies have shown that OGF is autocrine and paracrine produced in tissues originating from dermal derivatives, with brain and gut tissues having the greatest levels of the peptide[12,13].

The inhibitory effects of OGF on cell replication were first recorded in developing rat brain[14,15] and in tissue culture studies on mouse and human neuroblastoma[16-19]. OGF inhibits DNA synthesis and cell replication of normal cells and tissues[20,21], human neoplasia[22], and bacteria[23]. The main action of OGF is to upregulate inhibitory kinases in the cell cycle process. OGF's activity is receptor-mediated, dose-related, time-dependent, and reversible. The peptide is present in developing and renewing tissues, and has been localized in embryonic tissues and many human cancers[24-27].

OPIOID RECEPTORS

Classical opioid receptors were discovered in 1973 by three independent laboratories[28-30] and were identified in brain and gastrointestinal tissues. Based on their binding activities to radioisotopes, three classes of receptors - mu (MOR), delta (DOR), and kappa (KOR) were characterized in membrane homogenates as 7-member trans-membrane cytoplasmic receptors. The gene and protein structures for the classical receptors are homologous, and many of the opioid ligands cross-react with more than one receptor. OGF binds to DOR and MOR receptors. However, a series of biochemical and pharmacological studies demonstrated that OGF also binds to a new opioid receptor, OGFr, that is located on the nuclear membrane[31-33]. Sequencing of OGFr reveals little or no homology with classical opioid receptors. However, OGFr does display pharmacological characteristics of opioid receptors such as stereospecificity of ligands and opioid antagonist blockade[34]. Subcellular fractionation studies of OGFr in developing rat brain and neuroblastoma cells reveal that OGFr is associated with the nuclear membrane, and immunoelectron microscopy studies have shown that OGF co-localizes with OGFr on the outer nuclear membrane and within the nucleus[35].

OGF-OGFR AXIS: PRECLINICAL *IN VITRO* STUDIES

OGF and OGFr are present in human pancreatic cells (PANC-1, MIA PaCa-2, BxPC-3 and Capan-1) grown in culture, xenografted to nude mice, or surgical specimens obtained during tumor resection[36-39]. *In vitro* studies using PANC-1 cells reveal that the receptor has specific and saturable binding affinity to radiolabeled [Met^5]-enkephalin, with enriched binding in the nuclear fraction of cells[38]. Competition experiments using ligands for classical opioid receptors [*e.g.*, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin] (DAMGO), [D-Pen^{2,5}]-enkephalin (DP-DPE), dynorphin A1-8, morphine] do not displace the affinity of [Met^5]-enkephalin for OGFr[38] supporting the selectivity of peptide and receptor.

The efficacy of OGF has been characterized in a series of tissue culture studies[37]. OGF inhibits DNA synthesis and the growth of PANC-1 cells in a dose-dependent (42% reductions) and temporal manner (up to 48% reductions at 120 h) relative to control cultures, with its action being receptor-mediated and reversible. Absorption of endogenous ligand using OGF antibodies negated growth inhibition associated with exogenous peptide administration. OGF has a ubiquitous effect on pancreatic cancer cells derived from tumors at different stages of differentiation. Administration of OGF for 72 h inhibits growth (up to 37%) of Capan-1 and MIA PaCa-2 cells, well-differentiated cancer cell lines[40,41], BxPC-3, a moderately well to poorly differentiated cell line[42], as well as PANC-1, an undifferentiated pancreatic cancer cell line[43].
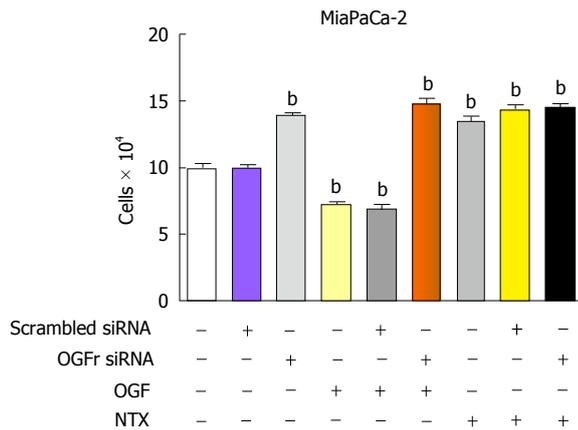
Figure 1 Opioid growth factor receptor is required for the inhibitory action of opioid growth factor and the stimulatory action of NTX on growth of MIA PaCa-2 human pancreatic cell cultures. Cells were transfected with opioid growth factor receptor (OGFr) siRNA or scrambled siRNA for 24 h and then treated with 10^{-6} mol/L. Opioid growth factor (OGF) or NTX, or 100 μL of sterile water for 72 h; compounds and media were replaced daily. Values are expressed as mean ± SE for cell counts from 2 aliquots/well and at least 2 wells/treatment. [b]P < 0.001 *vs* untransfected cultures.

Combinatorial therapeutic regimens are often more effective than single agent therapy. Gemcitabine is the standard of care for advanced pancreatic neoplasia, and also acts through inhibition of DNA synthesis[3]. Utilizing MIA PaCa-2 cells grown in log phase conditions, the combination of OGF (10^{-6} mol/L) and gemcitabine (10^{-8} mol/L) reduces cell number from control levels by more than 45% over 48 h, whereas each compound alone inhibits growth by less than 22% in the same time period. The action of OGF, but not gemcitabine, is mediated by a naloxone-sensitive receptor and is reversible. Combining OGF with 5-fluorouracil (5-FU; 10^{-6} mol/L) also increases inhibitory action. Over a 96 h period, OGF and 5-FU reduce cell number up to 30%, whereas each compound alone reduces cell number by up to 18%[39].

The specificity of OGF and OGFr has been documented in a variety of experiments using different human pancreatic cancer cell lines. The specificity of OGF has been confirmed by addition of multiple ligands that are specific for classical opioid receptors yet have no effect on cell proliferation and/or growth of cells or tumors[37-39]. Absorption of OGF by antibodies to the endogenous peptide depresses growth, demonstrating the specificity of this peptide. Competition binding assays using classical ligands such as DAMGO, DPDPE, morphine, ethylketocyclazocine and others results in no loss of binding of OGF to OGFr, suggesting little or no affinity of other ligands for OGFr[38].

OGFr selectivity and specificity for the ligand OGF have been shown in several experiments. In tissue culture, siRNA knockdown of RNA and protein expression of OGFr results in cultures that grow faster than controls because there is no receptor available for interaction with endogenous inhibitory OGF. Addition of exogenous OGF to cultures lacking OGFr has no inhibitory action (Figure 1). Finally, transient transfection experiments

that knockdown classical opioid receptors using siRNAs to MOR, DOR, or KOR results in no alteration in cell proliferation in homeostatic conditions or following the addition of OGF[44].

IN VIVO STUDIES ON OGF INHIBITION OF PANCREATIC TUMOR GROWTH

Transplantation of BxPC-3 human pancreatic cancer cell lines into nude mice has established a model to study how OGF inhibits tumor incidence and growth[36]. Athymic mice were inoculated subcutaneously with BxPC-3 cells and injected intraperitoneally 3 times daily with 5 mg/kg OGF or sterile saline. OGF-treated mice exhibited a delay of 43% in the time of initial tumor appearance relative to controls (10.6 d). Importantly, 62% of the OGF-treated mice did not have tumors on the day when 100% of all saline-injected mice had visible tumors, suggesting that OGF inhibits proliferation of tumor cells in such a way as to prevent tumor appearance. For those mice receiving OGF that did develop tumors, growth was markedly slower relative to mice injected with saline.

Studies utilizing MIA PaCa-2 cells were conducted in nude mice receiving combination therapy of OGF and/or gemcitabine[39]. Measurement of human pancreatic tumor growth (MIA PaCa-2) in nude mice revealed marked reductions in tumor progression under all treatment modalities, but combination therapy resulted in tumors with marked reductions in size relative to control mice, as well as mice receiving either OGF alone (10 mg/kg daily) or gemcitabine alone (120 mg/kg every 3rd day) (Figure 2). Tumor volumes after 45 d of treatment were reduced approximately 83% in the combination therapy group relative to controls (8900 mm³), whereas reductions in tumor volume were 45% and 56% for mice receiving OGF alone or gemcitabine alone, respectively, relative to controls.

The relationship between OGFr levels and the progression of human pancreatic tumors in nude mice was investigated by assaying OGFr binding activity and *OGFr* gene expression in tumors of small, medium, or large volume[45]. Binding capacity of OGFr, and transcriptional activity of OGFr, were not dependent on the size of tumor and were unaltered between small and large tumors. Interestingly, OGF plasma levels were decreased up to 7.9-fold in untreated mice with tumors relative to normal, non-tumorigenic mice suggesting that production of the inhibitory peptide, but not the receptor, may be deficient as cancer progresses[45]. These data support that exogenous administration of OGF is important as a therapy because the receptor is present and functioning in late-stage mouse tumors.

Overexpression of OGFr in tumor cells transplanted into nude mice confirmed that the OGF-OGFr axis provides tonic, homeostatic regulatory control of pancreatic neoplasia[46,47]. MIA PaCa-2 cells were stably transfected to overexpress OGFr, selectively cloned and expanded, and inoculated into nude mice; phenotypic changes in tumorigenicity were monitored. Analysis of receptor
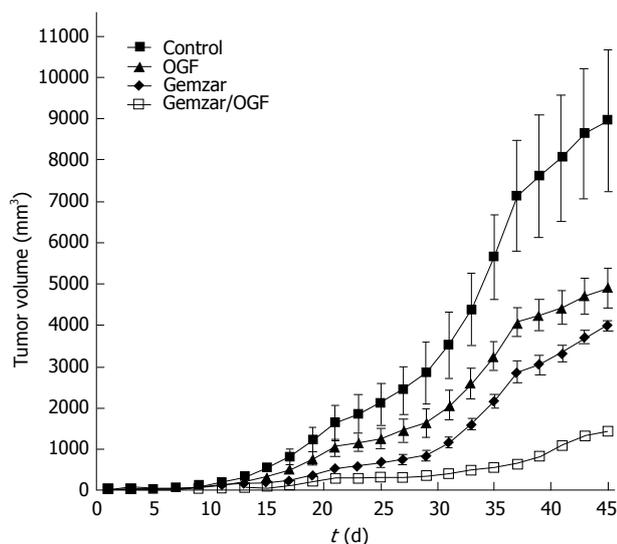
Figure 2 Tumor growth of MIA PaCa-2 tumors xenografted into nude mice. Animals were injected with 10 mg/kg opioid growth factor (OGF) daily, 120 mg/kg gemcitabine every 3 d (gemzar), both OGF and Gemzar, or 0.1 mL of sterile saline daily (control). Tumor volumes were monitored with calipers over a 45-d period of time. Values represent mean ± SE for all mice in the group. See original manuscript[39] for statistical comparisons.

number showed that transfected tumor tissue had more than 4 times the binding capacity compared to wildtype tumors. Tumor incidence in mice receiving the molecularly manipulated cells was reduced up to 50% from animals inoculated with wildtype or empty vector transfected cell lines. Latency for the appearance of a measurable tumor was increased 30%, whereas tumor volumes were decreased 70% in comparison to measurements in mice receiving cells transfected with empty vector cDNA constructs. Treatment of mice with an overexpression of OGFr reduced tumor volumes even more with reductions up to 55% recorded[47]. Therefore, OGFr is a regulator of neoplastic cell proliferation that impacts human pancreatic tumorigenic expression. Modification of receptor number alone may prevent or delay human pancreatic cancer.

OGF-OGFR AXIS: MECHANISM OF ACTION

The mechanism of action of OGF is targeted to DNA synthesis and is directed to the p21 cyclin-dependent inhibitory kinase pathway in human pancreatic cancer[37,48,49]. OGF action is mediated by the receptor OGFr. Unlike the mechanistic pathways of many of the standard chemotherapies, investigations have shown that OGF is nontoxic and does not induce apoptosis[50]. Using a variety of human cancer cell lines, studies have demonstrated that OGF does not reduce cell number by changing other biological pathways associated with migration, differentiation, or cell death[50-52]. Flow cytometric analyses of BxPC-3 cell lines treated with OGF reveal a notable increase in cell number in the G_0/G_1 phase and compensatory reduction in the proportion of cells in the S and G_2/M phases. The percentage of labeled mitotic cells was

increased in the G_0/G_1 phase[48]. Further studies utilizing synchronized cultures of BxPC-3 pancreatic cells were directed at deciphering the specific pathway in the cell cycle that is targeted by OGF and focused on the retinoblastoma pathway[49]. It was found that OGF decreased phosphorylation of retinoblastoma protein, but did not change the overall level of retinoblastoma protein. The change was correlated with a reduction in cyclin dependent kinase activity (cdk-2), and increased p21 expression[49]. In general, human pancreatic cancer cells express p21 cyclin-dependent inhibitory kinase pathways, whereas many other cancers (*e.g.*, squamous cell carcinoma of the head and neck) utilize p16 cyclin-dependent inhibitory kinase pathways because of deletions or mutations in the p21 pathway. The presence of one intact pathway is important to maintain a homeostatic balance of cellular replication, allowing for one pathway to be mutated as is often the case in neoplasia. The requirement of an intact OGF-OGFr axis for regulation of pancreatic neoplasia was corroborated in a study[44] whereby more than 30 human cancer cell lines were transfected to repress OGFr cDNA and OGFr expression. The lack of OGFr rendered OGF ineffective in inhibiting proliferation.

CLINICAL STUDIES ON THE SAFETY AND EFFICACY OF OGF FOR TREATMENT OF HUMAN PANCREATIC CANCER

Preclinical studies on OGF have shown no toxicity and significant efficacy toward repressing pancreatic cancer progression. Clinical trials to assess OGF treatment of advanced pancreatic cancer were conducted by Zagon *et al*[52] and Smith *et al*[53] at The Pennsylvania State University College of Medicine. The maximum tolerated dose (MTD) was established at 250 μg/kg infused over a period of 30 min[52]. Patients with unresectable advanced pancreatic adenocarcinoma were treated with the MTD to establish safety and toxicity. No adverse effects related to cardiac rhythm, blood values, neurological status or other laboratory tests were reported; hypotension was the dose-limiting toxicity. Of interest were the signs of efficacy shown by the small number of patients in this phase I trial. Mean survival time for the patients in the study, including those receiving only one dosage of OGF, was over 8.5 mo, and two patients had resolution of liver metastases. These observations support further clinical trials on OGF as a treatment of advanced pancreatic cancer.

A prospective phase II open-labeled clinical trial with 24 patients who failed standard chemotherapy for advanced pancreatic cancer was conducted whereby patients were treated weekly with 250 μg/kg OGF by intravenous infusion[54]. Outcomes were tumor size measured by computer tomography, survival time, and quality of life. Blood samples were evaluated for levels of OGF after 4 and 8 wk of infusion. Data on the OGF treatment were compared to results obtained from a control group (n-166) of patients of equivalent age who failed therapy

and were discharged to hospice care. OGF-treated patients had a three-fold increase in median survival time in comparison to untreated patients. Tumor size was stabilized or reduced in 62% of the cancer patients receiving OGF and surviving more than 8 wk in order to conduct the tomography. Plasma enkephalin levels were significantly increased at 4 and 8 wk with blood levels reaching approximate 55 pg/mL in comparison to baseline values of 8 pg/mL. Finally, no adverse effects on blood chemistry were noted, confirming the safety and lack of toxicity of OGF. Feedback from patients receiving OGF and their caregivers on quality of life indicated that OGF infusion did not indicate any stress or pain.

OGF BIOTHERAPY

Preclinical studies using a variety of human pancreatic adenocarcinoma cell lines that represent undifferentiated to well differentiated pancreatic neoplasms have demonstrated that OGF inhibits DNA synthesis and cell proliferation *in vitro*. The action of OGF is mediated by OGFr, is reversible, and does not involve apoptotic pathways. OGF is an endogenous peptide that is readily degraded, without alteration of cell migration, differentiation, or survival and thus can be considered a biotherapy. The specificity and selectivity of the OGF-OGFr axis substantiate that this axis is a determinant of cell proliferation in a variety of human cancers.

Investigations of the OGF-OGFr axis in mouse models of cancer with human cell lines transplanted into nude mice confirmed and extended tissue culture studies. Exogenous OGF repressed tumor progression under all situations, and tumors grown from cells overexpressing OGFr were inhibited in their growth. Combination OGF and chemotherapy provided enhanced efficacy at reducing tumor size.

Clinically, OGF is a safe, non-toxic biotherapy that extends survival and reduces tumor burden in patients with unresectable pancreatic cancer. In summary, the OGF-OGFr axis should be explored both as a primary therapy for pancreatic cancer, and as an adjuvant pathway with other chemotherapies.

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Beyond first-line chemotherapy for advanced pancreatic cancer: An expanding array of therapeutic options?

Evan J Walker, Andrew H Ko

Evan J Walker, Andrew H Ko, University of California, Helen Diller Family Comprehensive Cancer Center, San Francisco, CA 94115, United States
Author contributions: Walker EJ performed the literature search; Walker EJ and Ko AH designed and wrote the paper.
Correspondence to: Andrew H Ko, MD, Associate Professor of Medicine, University of California, Helen Diller Family Comprehensive Cancer Center, 1600 Divisadero St, Box 1705, San Francisco, CA 94115,
United States. andrewko@medicine.ucsf.edu
Telephone: +1-415-3537286 Fax: +1-415-3537984
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Abstract

While an increasing number of therapeutic options are now available for the first-line treatment of locally advanced or metastatic pancreatic cancer, the optimal choice for treatment in the second-line setting and beyond is less well defined. A variety of cytotoxic agents, either alone or in combination, have been evaluated, although primarily in the context of small single-arm or retrospective studies. Most regimens have been associated with median progression-free survival rates in the range of 2-4 mo and overall survival rates between 4-8 mo, highlighting the very poor prognosis of patients who are candidates for such treatment. Targeted therapies studied in this chemotherapy-refractory setting, meanwhile, have produced even worse efficacy results. In the current article, we review the clinical evidence for treatment of refractory disease, primarily in patients who have progressed on front-line gemcitabine-based chemotherapy. In the process, we highlight the limitations of the available data to date as well as some of the challenges in designing appropriate clinical trials in this salvage setting, including how to select an appropriate control arm given the absence of a well-established reference standard, and the importance of incorporating predictive biomarkers and quality of life measures whenever possible into study design.

Core tip: No standard of care exists for patients with advanced pancreatic cancer who have progressed on front-line chemotherapy. To date, most available evidence has come from small non-randomized studies, with efficacy results that have been fairly dismal. In this review, we discuss both traditional and novel cytotoxic and targeted therapies that have been evaluated in this refractory setting and how they may (or may not) be applicable to clinical practice; and raise considerations for clinical trial design in the future, particularly in this current era of both expanding chemotherapeutic options and molecular/"precision" medicine.

INTRODUCTION

More than 80% of patients diagnosed with pancreatic adenocarcinoma have metastatic or locally advanced inoperable disease at the time of initial presentation[1], at which point systemic therapy becomes the mainstay of care. Over the past decade-plus, gemcitabine alone or in combination with other drugs (most commonly a fluoropyrimidine, a platinum analogue, or the epidermal growth

factor receptor inhibitor erlotinib) have represented the most commonly used front-line treatment options. The treatment landscape is gradually shifting, however, with recent positive results from a couple of phase III studies establishing two new standards of care for first-line treatment, FOLFIRINOX [infusional 5-fluorouracil (FU), leucovorin, irinotecan, oxaliplatin] and the doublet of gemcitabine plus *nab*-paclitaxel.

Invariably, regardless of choice of front-line therapy, patients with advanced/metastatic disease will progress, and at that point the choice of treatment becomes considerably murkier. According to results from one United States cooperative group trial (CALGB 80303), fewer than half of patients with advanced pancreatic cancer went on to receive any additional therapy after progressing on front-line study treatment[2]. This reflects, in part, the fact that patients in this setting frequently demonstrate significant clinical deterioration and a decline in performance status, and are no longer deemed appropriate candidates for further anti-cancer therapy. However, it also highlights the fact that no second-line regimen(s) has consistently and unequivocally been shown to confer a survival benefit for patients, and as such providers are left grasping for best available evidence to inform treatment decisions, especially for patients who wish to remain proactive with some form of therapy.

In this review, we summarize the various therapeutic options that have been evaluated to date in the second-line (and beyond) setting for advanced pancreatic cancer. In so doing, we raise a number of important issues regarding appropriate clinical trial design, what (if any) should be considered a correct reference standard and benchmark of success in this setting, and how the expanding armamentarium of available agents and established regimens for this disease both expands our array of therapeutic options and adds to the complexity in decision-making.

GEMCITABINE-CONTAINING REGIMENS

Gemcitabine emerged as the standard of care for first-line treatment of advanced pancreatic cancer following its FDA approval in 1996[3]. Once patients develop resistance following front-line gemcitabine-based therapy, the natural question arises as to whether continuing with this same drug while adding novel agents can confer, or restore, clinical activity by overcoming drug-specific chemotherapeutic resistance and/or through synergistic effects.

Kozuch *et al*[4] first demonstrated the feasibility of this approach in a retrospective analysis of 34 consecutive patients with metastatic pancreatic cancer receiving irinotecan/gemcitabine/5-FU/leucovorin/cisplatin (G-FLIP), 32 of whom had previously progressed on gemcitabine and 31 who had progressed specifically on gemcitabine/5-FU/cisplatin (GFP). Of these 31 patients, whose regimen was altered only by the addition of irinotecan, 7 (23%) achieved partial responses (PR) and 7

(23%) achieved stable disease (SD). Notably, 8 of these 14 patients demonstrating disease control had previously experienced progressive disease as a best response to GFP alone. Median progression-free and overall survival (OS) for all 34 patients receiving second-line G-FLIP was 3.9 and 10.3 mo, respectively.

Another multidrug regimen that has been evaluated in the refractory setting is cisplatin/epirubicin/5-FU/gemcitabine (PEFG). This combination was initially tested in the front-line setting in an Italian phase III trial by Reni *et al*[5], and showed improved 4-mo PFS and 2-year survival rates compared to gemcitabine monotherapy, albeit with significant rates of hematologic toxicity. PEFG was subsequently studied by the same research group as second-line therapy in patients with progressive or metastatic disease refractory to gemcitabine-based treatment. In this 46-patient study, subjects receiving either classic or dose-intense PEFG had a median OS of 8.3 mo, with no significant difference between the different doses of PEFG tested[6]. Again, marked toxicities were noted, including Grade 3-4 neutropenia and thrombocytopenia in 26 (56%) and 10 (22%) patients, respectively.

Building upon observations from prior phase III trials demonstrating improvements in response rate (RR), progression free survival (PFS), and clinical benefit response (CBR) of gemcitabine/platinum doublets compared to gemcitabine monotherapy in the front-line setting[7,8], a similar strategy has also been explored in the gemcitabine-refractory setting in a variety of contexts. Demols *et al*[9] investigated the combination of gemcitabine plus oxaliplatin (GemOx) in a single-arm phase II study involving 33 patients with gemcitabine-refractory advanced pancreatic cancer. A partial response was observed in 7 patients (21%) with an additional 12 patients (36%) achieving SD. Median OS was 6 mo. Importantly, 17 patients (52%) were reported as having a clinical benefit response. One more recent approach has involved testing the potential for enhanced chemotherapeutic efficacy at higher temperatures[10], by which basis Tschoep-Lechner *et al*[11] conducted a study of gemcitabine and cisplatin combined with regional hyperthermia (RHT) in the second-line setting. Median time to progression for the 23 patients treated with this strategy was 4.3 mo, with a median overall survival of 12.9 mo. These results have spurred an ongoing prospective phase II trial offering second-line Gem/Cis/RHT (EudraCT: 2005-003855-11).

Other doublet regimens that have been evaluated in the salvage setting include gemcitabine plus the oral fluoropyrimidine S-1[12] and gemcitabine plus *nab*-paclitaxel[13] with median times to progression of 2.8 and 3.2 mo, respectively. More details of these and other gemcitabine-based combinations are summarized in Table 1.

NOVEL MONOTHERAPEUTIC REGIMENS

An alternative approach to second-line therapy involves administration of a completely non-cross-resistant regimen; using such a strategy, previous agents (such as

Table 1 Clinical studies of second-line gemcitabine-containing regimens						
Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Kozuch *et al*[4], 2001	G-FLIP	34	24%	3.9	10.3	47%
Reni *et al*[6], 2008	PEFG	46	24%	5.0	8.3	26%
Demols *et al*[9], 2006	GEMOX	33	21%	4.2	6.0	NR
Fortune *et al*[76], 2009	GEMOX	17	24%	2.6	6.4	29%
Stathopoulos *et al*[77], 2006	Gem, Lipoplatin	24	8.3%	NR	4.0	NR
Tschoep *et al*[11], 2013	Gem, Cisplatin, RHT	23	4.3%	4.3	NR	NR
Morizane *et al*[12], 2012	Gem, S-1	40	18%	2.8	7.0	18%
Ernani *et al*[13], 2012	Gem, *nab*-Paclitaxel	10	20%	3.2	NR	NR

[1]Intent-to-treat analysis. G-FLIP: Gemcitabine, 5-fluorouracil, leucovorin, cisplatin; PEFG: Cisplatin, epirubicin, 5-fluorouracil, gemcitabine; GEMOX: Gemcitabine, oxaliplatin; Gem: Gemcitabine; RHT: Regional hyperthermia; *Nab*-paclitaxel: Albumin-bound nanoparticle paclitaxel; NR: Not reported; PFS: progression free survival; OS: Overall survival; TTP: Time to progression.

gemcitabine) are discontinued and an entirely new drug or drug combination is given. In terms of monotherapy, several topoisomerase inhibitors have been investigated in patients refractory to gemcitabine-based front-line treatment. The orally active camptothecin rubitecan, for example, showed sufficient single-agent activity in two separate studies of gemcitabine-refractory disease[14,15] to warrant a randomized phase Ⅲ trial in which 409 pretreated patients (70% of whom had received two or more prior regimens) were randomized to receive either rubitecan monotherapy or "best choice (BC)" alternative therapy as determined by treating physicians (most commonly gemcitabine, 5-FU, mitomycin C, capecitabine, or docetaxel). Presented as an abstract at the 2004 ASCO annual meeting but never subsequently published, the trial did not show a statistically significant difference in overall survival between groups (108 d *vs* 94 d, respectively, *P* = 0.63), although significant improvements were observed with rubitecan in terms of progression-free survival (58 d *vs* 48 d, *P* = 0.01) and response rate (6.1% *vs* 0.5%, *P* = 0.01)[16].

More recently, a phase Ⅱ study of liposomal irinotecan sucrosofate (PEP02, MM-398), a drug formulation with improved pharmacokinetics and tumor bioavailability relative to free irinotecan, was performed in patients with metastatic pancreatic cancer refractory to front-line gemcitabine-based therapy[17]. Ko *et al*[17] reported a disease control rate of 50% (including 7.5% with an objective response) as well as a 50% or greater CA19-9 decline in 31% of evaluable subjects, with a median overall survival of 5.2 mo. Toxicities were manageable, with cytopenias, asthenia, and diarrhea representing the most common grade 3/4 adverse events. These results prompted the launch of an international randomized phase Ⅲ trial (NAPOLI-1, NCT01494506) that has been recently completed, comparing MM-398 with or without 5-FU/leucovorin to 5-FU/leucovorin alone.

Inhibitors of microtubule dynamics, including taxanes (docetaxel, paclitaxel, *nab*-paclitaxel) and eribulin mesylate, have also been investigated in small retrospective and single-arm phase Ⅱ studies[18-22]. Given the unique formulation of *nab*-paclitaxel that may allow it to more successfully traverse the blood-stroma barrier, in addition to the positive results from the phase Ⅲ MPACT trial es-

tablishing the combination of *nab*-paclitaxel/gemcitabine as a viable option for first-line therapy[23], there has been natural interest in evaluating this agent in the salvage setting. To date, we only have results from a small phase Ⅱ study of *nab*-paclitaxel as a single agent for refractory pancreatic cancer, in which there was a single objective response (with an additional 6 achieving disease stabilization) amongst 19 patients, with a median PFS of 1.7 mo. Estimated median OS in this cohort was 7.3 mo[22].

Fluoropyrimidines have also been studied in the advanced refractory disease setting. Boeck *et al*[24] studied second-line capecitabine monotherapy after gemcitabine failure and observed disease stabilization in 39% of patients (no objective responses), with a median time to progression and overall survival of 2.3 mo and 7.6 mo, respectively. Another oral fluoropyrimidine, S-1, widely used in Asia and other parts of the world for gastric and pancreatic cancer, has also been evaluated in several phase Ⅱ studies as monotherapy for gemcitabine-refractory patients; response rates associated with this agent range from 4%-15%, with a median PFS almost uniformly in the 2 mo range[25-28]. See Table 2 for additional data from these studies.

CYTOTOXIC COMBINATION REGIMENS (NON-GEMCITABINE-BASED)

Patients who maintain a good performance status after progressing on front-line therapy may also be candidates for non-gemcitabine-based combination chemotherapy regimens.

Platinum-based combinations

To date, the majority of studies have concentrated on the combination of a fluoropyrimidine plus a platinum analogue, most notably 5-FU, leucovorin, and oxaliplatin administered in various dosing schedules. One of the earliest studies, a non-randomized phase Ⅱ trial conducted in Greece by Tsavaris *et al*[29], showed encouraging clinical activity of these drugs when administered weekly in bolus fashion, with the best response including partial responses in 7 of 30 patients (23%) and stable disease in an additional 9 (30%). More traditional FOLFOX regimens, with biweekly dosing schedules and prolonged 5-FU infusion

Table 2 Clinical studies of second-line monotherapeutic regimens

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Jacobs *et al*[16], 2004	Rubitecan	198	11%	1.9	3.5	NR
Burris *et al*[15], 2005	Rubitecan	58	5.2%	2.0	3.1	9%
Yi *et al*[78], 2009	Irinotecan	33	9%	2.0	6.6	NR
Takahara *et al*[79], 2013	Irinotecan	56	3.6%	2.9	5.3	NR
Ko *et al*[17], 2013	Nanoliposomal irinotecan	40	7.5%	2.4	5.2	25%
Oettle *et al*[18], 2000	Paclitaxel	18	5.6%	NR	4.1	NR
Maeda *et al*[19], 2011	Paclitaxel	30	10%	NR	6.7	NR
Cereda *et al*[20], 2008	Docetaxel	10	0%	1.5	4.0	0%
Hosein *et al*[22], 2013	*Nab*-Paclitaxel	19	5%	1.7	7.3	37%
Boeck *et al*[24], 2007	Capecitabine	39	0%	2.3	7.6	NR
Bodoky *et al*[59], 2012	Capecitabine	38	7.9%	2.2	5.0	NR
Morizane *et al*[25], 2009	S-1	40	15%	2.0	4.5	14%
Todaka *et al*[26], 2010	S-1	52	3.8%	2.1	5.8	12%
Mizuno *et al*[28], 2013	S-1	67	6%	1.9	5.9	NR
Ioka *et al*[27], 2013	Best fluoropyrimidine[2]	40	10%	3.8	7.5	NR
Fukahori *et al*[80], 2012	Gemcitabine[3]	27	14%	2.6	8.0	NR
Androulakis *et al*[81], 2005	Oxaliplatin	18	0%	NR	3.5	NR
Boeck *et al*[82], 2007	Pemetrexed	52	3.8%	1.6	4.7	NR
Ulrich-Pur *et al*[48], 2003	Raltitrexed	19	0%	2.5	4.3	0%
Kindler *et al*[83], 2008	Arsenic trioxide	13	0%	1.6	3.8	0%

[1]Intent-to-treat analysis; [2]S-1 (67.5%), uracil-tegafur (20%), or 5-fluorouracil (12.5%); [3]S-1 refractory disease. *Nab*-paclitaxel: Albumin-bound nanoparticle paclitaxel; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

times similar to that given in colorectal cancer, have also been examined with demonstrable evidence of activity in this setting. Yoo *et al*[30] conducted a randomized phase II trial comparing modified versions of FOLFOX and FOLFIRI (5-FU, leucovorin, irinotecan) for gemcitabine-refractory advanced pancreatic cancer. However, in this study, response rates to both regimens were low (7% and 0%) with associated PFS times of 6.0 and 8.3 wk, respectively. A more recent phase II trial of FOLFOX4 from Korea reported modestly better results, with an objective response rate of 11%, a tumor stabilization rate of 41%, and a median time to progression of 9.9 wk[31]. Single-arm studies of capecitabine plus oxaliplatin (CapOx) have also been performed by several Asian groups, with fairly comparable results[32-35].

The most convincing evidence supporting a fluoropyrimidine/platinum-based combination comes from Germany, using a regimen termed OFF, in which 5-FU (given as a 24-h infusion) plus folinic acid are given weekly x 4 in 6-wk cycles, with the addition of oxaliplatin during weeks 2 and 4. Prompted by promising results from a phase II trial using this regimen (disease control rate lasting 12 wk or better in 43% of study patients), a phase III randomized trial was designed by Charité Onkologie (CONKO-003) in which patients were randomized to receive either the OFF regimen or best supportive care (BSC). A sample size of 165 was planned, but the study was stopped due to poor accrual (likely from the possibility of randomization to a BSC arm) after enrolling 46 patients[36]. Even with the limited sample size, overall survival in patients receiving OFF was 4.8 mo compared to 2.3 mo in those receiving BSC (*P* = 0.008)[37]. The investigators sought to build on these results with another randomized phase III trial comparing OFF to weekly 5-FU/folinic acid (FF) alone. The results of this

168-patient trial were presented in abstract form at the 2008 ASCO meeting[38]. As compared to the FF regimen, patients receiving OFF demonstrated improved PFS (13 wk *vs* 9 wk, *P* = 0.012) and median OS (26 wk *vs* 13 wk, *P* = 0.014). This trial marks the largest phase III study to date showing a survival benefit of second-line therapy for pancreatic cancer; as such, the OFF regimen (or iterations thereof) has become accepted as the de facto standard treatment of refractory disease.

With the emergence of FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin) as a front-line standard for patients with advanced pancreatic cancer and good performance status[39], there has naturally been interest in investigating this regimen in the second-line setting. To date, we only have data from one small retrospective series that included 27 patients[40]. Seventeen (63%) demonstrated stable disease or better, including 5 with partial responses, with an associated median TTP of 5.4 mo. Importantly, treatment was generally well-tolerated with manageable and predictable toxicities. Further evaluation of this regimen clearly needs to be performed in prospectively designed studies.

While fluoropyrimidine/platinum combinations have been studied most extensively, single-arm studies of platinum-based agents partnered with other classes of agents, including oxaliplatin in combination with irinotecan[41,42], raltitrexed[43], and pemetrexed[44], have also been examined. Results of these small series are shown in Table 3.

Non-platinum-based combinations
In addition to the previously described phase II trial by Yoo *et al*[30] in which gemcitabine-refractory patients were randomized to receive modified versions of either FOLFOX or FOLFIRI, other smaller prospective and retrospective studies of FOLFIRI have been conducted,

Table 3 Clinical studies of second-line cytotoxic combination regimens

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
	Platinum based regimens					
Tsavaris *et al*[29], 2005	FOLFOX	30	23%	5.1	5.8	NR
Mitry *et al*[84], 2006	FOLFOX	18	0%	0.9	1.3	NR
Gebbia *et al*[85], 2007	FOLFOX	42	14%	4	6.7	NR
Novarino *et al*[86], 2009	FOLFOX	23	0%	2.7	4.0	NR
Yoo *et al*[30], 2009	FOLFOX	30	6.7%	1.4	3.5	NR
Chung *et al*[31], 2013	FOLFOX	44	11%	2.3	7.3	NR
Berk *et al*[35], 2012	FOLFOX	46	17%	3.7	5.8	NR
Sancho *et al*[32], 2008	CapOx[2]	18	5.6%	3.9	5.8	NR
Xiong *et al*[33], 2008	CapOx	41	2.4%	2.3	5.4	21%
Gasent-Blesa *et al*[34], 2009	CapOx	15	6.7%	NR	5.3	NR
Berk *et al*[35], 2012	CapOx	39	18%	3.7	4.9	NR
Pelzer *et al*[87], 2009	OFF	37	5.4%	2.8	5.1	NR
Pelzer *et al*[37], 2011	OFF	23	0%	NR	4.8	NR
Pelzer *et al*[38], 2008	OFF	76	NR	3	6.1	NR
Assaf *et al*[40], 2011	FOLFIRINOX	27	19%	5.4	8.5	NR
Togawa *et al*[88], 2007	Cisplatin, S-1	17	29%	NR	10	32%
Kim *et al*[89], 2012	Cisplatin, S-1	11	0%	1.5	2.7	NR
Takahara *et al*[90], 2013	Oxaliplatin, S-1	30	10%	3.4	5.0	NR
Cantore *et al*[41], 2004	Oxaliplatin, irinotecan	30	10%	4.1	5.9	23%
Oh *et al*[42], 2010	Oxaliplatin, irinotecan	14	21%	1.4	4.1	7.1%
Reni *et al*[43], 2006	Oxaliplatin, raltitrexed	41	24%	1.8	5.2	12%
Mazzer *et al*[44], 2009	Oxaliplatin, pemetrexed	16	56%	3.3	NR	NR
	Non-platinum based regimens					
Yoo *et al*[30], 2009	FOLFIRI	31	0%	1.9	3.9	NR
Gebbia *et al*[45], 2010	FOLFIRI	40	15%	3.7	6.0	0%
Cereda *et al*[91], 2010	FOLFIRI or XELIRI	34	0%	2.0	4.2	5.6%
Zaniboni *et al*[46], 2012	FOLFIRI	50	8%	3.2	5.0	NR
Neuzillet *et al*[47], 2012	FOLFIRI	63	7.9%	3.0	6.6	NR
Mizuno *et al*[28], 2013	S-1, irinotecan	60	18%	3.6	6.9	NR
Blaya *et al*[49], 2007	Capecitabine, docetaxel	24	13%	NR	NR	NR
Katopodis *et al*[50], 2011	Capecitabine, docetaxel	31	9.7%	2.4	6.4	15%
Kim *et al*[51], 2009	5-FU, paclitaxel	28	10%	2.5	7.6	NR
Lee *et al*[92], 2009	Conti-FAM[3]	31	12%	2.3	6.7	NR
Shi *et al*[93], 2012	Capecitabine, thalidomide	31	6.5%	2.7	6.1	NR
Saif *et al*[94], 2009	Capecitabine, PHY906	25	5.3%	NR	NR	NR
Ulrich-Pur *et al*[48], 2003	Irinotecan, raltitrexed	19	16%	4.0	6.5	NR
Reni *et al*[95], 2004	MDI	15	0%	1.7	6.1	0%
Cereda *et al*[96], 2011	Mitomycin, ifosfamide	21	4.8%	1.7	3.7	9.5%
Ko *et al*[52], 2008	Irinotecan, docetaxel	14	0%	1.2	4.5	21%

[1]Intent-to-treat analysis; [2]Pooled analysis of pancreatic (50%), biliary (22%), gallbladder (22%) and ampullary (6%) cancer; [3]Pooled analysis of pancreatic (48%), biliary (35%) and gallbladder (16%) cancer. FOLFOX: Oxaliplatin, 5-fluorouracil, folinic acid, biweekly; CapOx: Capecitabine, oxaliplatin; OFF: Oxaliplatin, 5-fluorouracil, leucovorin, in 6-wk cycles; FOLFIRINOX: Oxaliplatin, leucovorin, 5-fluorouracil, irinotecan; FOLFIRI: 5-Fluorouracil, leucovorin, irinotecan; XELIRI: Capecitabine, irinotecan; 5-FU: 5-Fluorouracil; Conti-FAM: 5-Fluorouracil, doxorubicin, mitomycin-c; MDI: Mitomycin, docetaxel, irinotecan; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

with response rates ranging between 8%-15% and median progression-free survival in the 3-4 mo range[45-47]. Another fluoropyrimidine/irinotecan combination termed IRIS (irinotecan plus S-1) was compared to S-1 alone in a randomized phase Ⅱ trial from Japan of 127 patients who had progressed on gemcitabine[28]. The combination produced a response rate of 18%, compared to 6% with S-1 alone (*P* = 0.03). Median PFS and OS also favored the IRIS combination, although these improvements did not reach statistical significance (107 and 208 d, compared to 58 and 176 d for S-1, respectively). Irinotecan has also been tested in combination with the folate antimetabolite raltitrexed in a randomized phase Ⅱ trial *vs* raltitrexed monotherapy[48]. In this 38-patient study, the doublet was associated with a higher rate of objective response (16% *vs* 0%) and prolonged PFS (4.0 mo *vs* 2.5

mo) and OS (6.5 mo *vs* 4.3 mo), albeit with higher rates of clinically relevant toxicities including gastrointestinal symptoms and alopecia.

Taxanes represent the other most frequently studied class of agents evaluated in the salvage setting for pancreatic cancer. Combination regimens including capecitabine/docetaxel[49,50] and 5-FU/paclitaxel[51] have been studied in small phase Ⅱ trials, with response rates in the 10% range and median PFS centered around 2 mo. A small phase Ⅱ study looking at the combination of irinotecan/docetaxel was discontinued early due to excess toxicity, with no responses observed in 14 evaluable patients[52]. Table 3 highlights other non-platinum-based combinations that have been explored, mostly in the context of single-arm phase Ⅱ studies.

Clinical trial	Design	Study arms	Goal enrollment	Primary measure	Previous therapy	Status
		Table 4 Ongoing randomized phase Ⅱ/Ⅲ trials of refractory pancreatic cancer chemotherapy				
NCT00674973	Phase Ⅱ	Erlotinib *vs* placebo	207	PFS, biomarkers	1 prior CT regimen	Active, not recruiting
NCT01074996	Phase Ⅱ	S-1 *vs* S-1, leucovorin	96	OS	Gem-based	Recruiting
NCT01417000	Phase Ⅱ	GVAX pancreas, cyclophosphamide, CRS-207 *vs* GVAX pancreas, cyclophosphamide	90	OS	≥ 1 prior CT regimen	Active, not recruiting
NCT01423604	Phase Ⅱ	Capecitabine, ruxolitinib *vs* capecitabine, placebo	138	OS	Gem-based	Active, not recruiting
NCT01658943	Phase Ⅱ	Selumetinib, MK2206 *vs* FOLFOX	133	OS, PFS	Gem-based	Recruiting
NCT01796782	Phase Ⅱ	QYHJ granules *vs* Capecitabine	60	OS	Non-capecitabine containing CT	Active, not recruiting
NCT01121848	Phase Ⅲ	Capecitabine or 5-FU, leucovorin *vs* XELOX or mFOLFOX-6	128	PFS	Gem-based	Active, not recruiting
NCT01494506	Phase Ⅲ	MM-398 *vs* MM-398, 5-FU, leucovorin *vs* 5-FU, leucovorin	405	OS	Gem-based	Active, not recruiting
NCT01954992	Phase Ⅲ	Glufosfamide *vs* 5-FU	480	OS	Gem-based	Recruiting
NCT01956812	Phase Ⅲ	Gemcitabine, IMMU-107 *vs* Gemcitabine, placebo	440	OS	2 prior CT regimens, ≥ 1 Gem-based	Not yet open for recruitment

GVAX pancreas: Allogeneic pancreatic cancer cell vaccine, induces GM-CSF production; CRS-207: Attenuated listeria monocytogenes vaccine, induces immune response to mesothelin; MK2206: Akt inhibitor; FOLFOX: 5-Fluorouracil, leucovorin, oxaliplatin; QYHJ: Qingyihuaji formulation; 5-FU: 5-Fluorouracil; XELOX: Capecitabine, oxaliplatin; mFOLFOX-6: Modified schedule 5-fluorouracil, leucovorin, oxaliplatin; MM-398: Liposomal irinotecan; IMMU-07: Yttrium-90 radiolabeled humanized monoclonal antibody against mucin1 (CD227); PFS: Progression free survival; OS: Overall survival; CT: Chemotherapy; Gem-based: Gemcitabine-containing chemotherapy regimen.

TARGETED THERAPIES

In recent years, an improved understanding of cancer biology has led to the development of targeted therapies intended to inhibit tumor-specific proteins or pathways instrumental in cellular proliferation and survival. These include small molecule inhibitors, which inhibit a specific intracellular protein or pathway; or engineered antibodies, designed to target proteins expressed preferentially on the tumor cell surface. In pancreatic cancer, a number of potentially actionable oncogenic pathways have been identified for which such targeted therapies have been developed and tested, many in the chemo-refractory setting, either alone or in combination with other targeted or cytotoxic agents.

Small molecule inhibitors that bind the intracellular tyrosine kinase (TK) domain of the human epidermal growth factor receptor (HER1/EGFR) block signaling through this pathway that controls aspects of DNA synthesis, cell proliferation, adhesion, and migration. Erlotinib, one such anti-EGFR TK inhibitor (TKI), was approved in the front-line setting for advanced pancreatic cancer based on a small but statistically significant improvement in median survival when added to gemcitabine in a randomized phase Ⅲ trial led by the National Cancer Institute of Canada[53]. When tested as monotherapy in the setting of gemcitabine-refractory disease in a (non-published) phase Ⅱ trial, erlotinib produced prolonged disease control (greater than 8 wk) in 10/40 evaluable patients, with a median time to progression of 1.6 mo and a median survival of 4.1 mo[54]. A randomized trial of erlotinib *vs* placebo (NCT00674973) has completed accrual with the goal of identifying biomarkers predictive of benefit to this agent (Table 4); data are not yet available. Another phase Ⅱ study tested erlotinib in combination with capecitabine in the refractory setting and pro-

duced somewhat better results, including a 10% objective response rate, a median PFS of 3.4 mo, and a median OS of 6.5 mo, with no associated grade 4 toxicities[55].

Downstream of EGFR is the protein encoded by the *KRAS* oncogene, which is mutated and hence constitutively activated in the vast majority of pancreatic cancers[56-58]. While KRAS itself has proved to be challenging as a druggable target, KRAS effector pathways such as the MAP (RAF/MEK/ERK) signaling cascade may be more amenable to pharmacologic inhibition. Bodoky *et al*[59] investigated selumetinib, a selective MEK1/2 inhibitor, in a randomized phase Ⅱ trial *vs* capecitabine for gemcitabine-resistant pancreatic cancer. Selumetinib, though well tolerated, did not improve survival relative to capecitabine monotherapy, with median PFS and OS times of 2.1 and 5.4 mo compared to 2.2 and 5.0 mo, respectively. Two of 32 patients on the selumetinib arm (6.3%) did achieve a (unconfirmed) partial response.

Several lines of preclinical evidence indicate that inhibition of MEK induces compensatory hyperactivation of a semi-parallel EGFR signaling pathway, the PI3K/AKT cascade[60], and that simultaneous blockade of multiple nodes leads to better anti-tumor activity. Ko *et al*[61] tested this approach of dual inhibition for refractory pancreatic cancer in a multicenter phase Ⅱ study, using the combination of selumetinib plus erlotinib. Although no objective responses were observed, 12 of 46 patients (26%) achieved stable disease for a minimum of 12 wk, and 38% of evaluable patients had a biomarker response (CA19-9 decline > 50%). Median OS on this study was 7.5 mo. An ongoing randomized phase Ⅱ study led by the Southwest Oncology Group (SWOG 1115) is comparing the combination of selumetinib plus the AKT inhibitor MK2206 to standard FOLFOX chemotherapy in patients who have progressed on front-line gemcitabine-based treatment (NCT01658943) (Table 4).

Table 5 Clinical studies of second-line targeted therapies

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Ignatiadis *et al*[97], 2006	Gefitinib, docetaxel	26	0%	2.1	2.9	NR
Brell *et al*[98], 2009	Gefitinib, docetaxel	41	2.4%	1.8	4.5	0%
Kulke *et al*[55], 2007	Erlotinib, capecitabine	30	10%	3.4	6.5	26%
Tang *et al*[54], 2009	Erlotinib	50	0%	1.6	4.1	6 m = 39%[3]
Iyer *et al*[99], 2010	Erlotinib	18	0%	1.4	3.1	NR
Bodoky *et al*[59], 2012	Selumetinib	32	6.3%	2.1	5.4	NR
Ko *et al*[61], 2013	Selumetinib, erlotinib	46	0%	2.6	7.5	NR
Wolpin *et al*[62], 2009	Everolimus	33	0%	1.8	4.5	NR
Garrido-Laguna *et al*[63], 2010	Sirolimus	31	0%	NR	NR	6 m = 26%[3]
Javle *et al*[64], 2010	Everolimus, erlotinib	16	0%	1.6	2.9	NR
Javle *et al*[64], 2010	Temsirolimus	5	0%	0.6	1.5	NR
Dragovich *et al*[68], 2008	Vatalinib	65	NR%	6 m = 14%[3]	6 m = 31%[3]	NR
O'Reilly *et al*[69], 2010	Sunitinib	77	1.4%	1.3	3.7	NR
Ko *et al*[67], 2010	Bevacizumab, erlotinib	36	2.8%	1.3	3.4	6 m = 22%[3]
Astsaturov *et al*[100], 2011	Bevacizumab	16	0%	1.4	5.5	NR
Astsaturov *et al*[100], 2011	Bevacizumab, docetaxel	16	0%	1.6	4.2	NR
Milella *et al*[73], 2004	Celecoxib, 5-FU	17	12%	1.9	3.5	NR
Pino *et al*[74], 2009	Celecoxib, capecitabine[2]	35	8.6%	4.0	4.4	NR
Starling *et al*[101], 2012	Imatinib, gem, oxaliplatin	27	7.4%	4.6	5.6	28%
Carvajal *et al*[102], 2009	Flavopiridol, docetaxel	10	0%	1.9	4.2	0%
Nallapareddy *et al*[103], 2010	Sarcatinib	19	0%	1.6	2.5	NR

[1]Intent-to-treat analysis; [2]Pooled analysis of pancreatic (86%) and biliary (14%) cancer; [3]6 m: 6 mo survival rate. 5-FU: 5-Fluorouracil; Gem: Gemcitabine; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

Among other effects, the EGFR/PI3K/AKT signaling cascade results in activation of the mammalian target of rapamycin (mTOR) protein kinase. mTOR plays a central role in cell growth and cell-cycle control, integrating mitogenic signals from various extracellular ligands including EGF, insulin, and insulin-like growth factor (IGF-1/2). Wolpin *et al*[62] tested the direct mTOR inhibitor everolimus in gemcitabine-resistant disease, but observed no objective responses and a disease control rate of only 21%, with a median PFS of 1.8 mo. A trial of sirolimus monotherapy, in which 75% of patients had received prior chemotherapy, similarly revealed minimal to no clinical activity[63]. Javle *et al*[64] tested a dual inhibition strategy of everolimus in combination with erlotinib in a small phase Ⅱ study, but this study was closed early due to futility.

In a separate (but not unrelated) category, anti-angiogenic strategies, primarily targeting vascular endothelial growth factor (VEGF) and its corresponding receptor (VEGFR), have been extensively studied in pancreatic cancer in both the front-line and salvage settings. The anti-VEGF monoclonal antibody bevacizumab, which did not improve survival when added to either gemcitabine[65] or erlotinib/gemcitabine[66] as first-line therapy in two large randomized phase Ⅲ studies, has also been explored in the refractory setting, with fairly minimal activity. A phase Ⅱ trial by Ko *et al*[67] examined the combination of bevacizumab and erlotinib in gemcitabine-refractory patients and reported a progression-free survival rate of 1.3 mo, with a median OS of only 3.4 mo. Oral TKIs directed against VEGFR have also been explored, including fairly large single-arm phase Ⅱ studies of vatalinib[68] and sunitinib[69]. Sunitinib, tested in the context of a cooperative group study (CALGB

80603), reported a single objective response amongst 77 patients (1.3%), a disease control rate of 22%, and progression-free and overall survival times of 1.3 and 3.7 mo, respectively. Interestingly, recent evidence suggests that pancreatic cancer, despite VEGF/VEGFR upregulation, is poorly vascularized relative to other tumors[70]. These data may help explain the minimal efficacy of anti-angiogenic therapy in pancreatic cancer.

Several other potential oncogenic pathways have been targeted in the second-line setting. Cyclooxygenase-2 (COX-2) is upregulated in pancreatic cancer[71], and its product prostaglandin-E can transactivate EGFR and promote tumor survival[72]. Celecoxib, a selective COX-2 inhibitor, has been tested in combination with fluoropyrimidines (5-FU or capecitabine) in second-line regimens and found to produce response rates of 9%-12% with very mild side effect profiles[73,74]. Ruxolitinib, an oral inhibitor of Janus kinase (JAK) signaling that is approved for use in myelofibrosis, has been evaluated as second-line therapy in combination with capecitabine in a randomized phase Ⅱ trial in patients with refractory pancreatic cancer (NCT01423604); this study has completed accrual as of mid-2013 and results are currently being awaited (Table 4). Data from other studies of targeted therapies are shown in Table 5.

DISCUSSION

There is presently no universally accepted standard of care for patients with advanced pancreatic cancer who have progressed on front-line therapy. As described above, with a few notable exceptions, the vast majority of studies conducted in this setting have been single-arm, single-institution trials with relatively modest sam-

ple sizes. Such non-randomized trials need to be carefully interpreted in light of their inherent selection bias; certainly, those patients who are well enough to consider salvage treatment may already have more favorable tumor biology that influences patient outcomes, including survival rates, independent of the specific choice of therapy.

This argument certainly lends itself in support of randomized phase Ⅱ/Ⅲ trials; studies that fit this category and remain open or are still actively recruiting (as of December 2013) are presented in Table 4. However, it should be recognized that the design and performance of randomized studies in this setting is particularly challenging. As the CONKO investigators observed, a control arm of best supportive care alone, while perhaps appropriate in many cases, is not a particularly attractive option to patients and may hinder study enrollment. But deciding on what the appropriate reference standard should be in a randomized study design, absent compelling evidence to support one regimen over another, is not a straightforward issue. For example, can a fluoropyrimidine alone (capecitabine, S-1, or 5-FU) be considered adequate as a control arm? Some might argue that there are adequate data indicating that a (fluoropyrimidine plus oxaliplatin) combination is clearly superior, and thus represents a more appropriate (and ethical) comparator for a randomized trial. But for a novel agent being evaluated in this setting, does comparing it alone to a reference standard of, for example, FOLFOX, provide adequate study equipoise?

It should also be noted that almost all of the studies detailed above were conducted in the pre-FOLFIRINOX era; as such, they primarily included patients who received a gemcitabine-based regimen as front-line therapy. It would seem logical that for a patient in the present time who receives FOLFIRINOX as first-line therapy, the next step would be to try a gemcitabine-based regimen (monotherapy, gemcitabine/*nab*-paclitaxel, or perhaps another gemcitabine-based combination). However, prospective randomized studies are still required to support this recommendation. Moreover, such FOLFIRINOX-treated patients would obviously not be appropriate for enrollment onto a study in which (s)he might be randomized to receive any of these same drugs, alone or in combination, as part of the control arm. Thus, looking ahead, one must consider the possibility that separate clinical trials should be developed in the second-line setting depending on patients' first-line treatment exposure.

These conundrums highlight only some of the challenges in designing clinical trials in this refractory setting for pancreatic cancer. The other major obstacle hindering progress is the lack of validated predictive biomarkers for this disease that could help inform treatment decisions, whether for conventional cytotoxics or for targeted agents. The track record for targeted agents in chemorefractory pancreatic cancer is particularly dismal, bringing to light the fact that, in the future, we need to be superselective in identifying the patients most likely to

benefit from a particular novel therapy, and to develop patient enrichment schemes in clinical trial design accordingly. However, obtaining adequate tumor tissue in this patient population for identifying and validating predictive molecular markers represents a substantial ongoing challenge.

We also propose that certain uniform study benchmarks be established to define "success" for a particular regimen and justify moving on to a larger phase Ⅲ study. A recent systematic review of 34 studies found a median survival for any second line regimen of 6 mo, compared to 2.8 mo for best supportive care alone[75]. With this in mind, thresholds of at least 6 mo for median OS, at a bare minimum, and 4 mo for median PFS, represent reasonable starting points that could be considered clinically meaningful and reflect treatment efficacy that matches or is superior to most historic data reported to date.

Additionally, cost-effectiveness analysis represents an important element to consider embedding within trial design, especially in larger studies, to help inform broader health care decisions in this clinical context in which the magnitude of survival benefit of any novel agent or regimen is likely to be measurable in extra months, if not only weeks. Finally, and perhaps even more importantly, we recommend that every effort should be made to incorporate quality of life (QoL) endpoints/patient-reported outcomes into study design, as these measures are of paramount importance for patients in this late-stage setting.

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2014 ADVANCES IN PANCREATIC CANCER

Pancreatic cancer stroma: Understanding biology leads to new therapeutic strategies

Agnieszka Anna Rucki, Lei Zheng

Agnieszka Anna Rucki, Lei Zheng, Department of Oncology, The Sidney Kimmel Cancer Center, the Skip Viragh Clinical Pancreatic Cancer Center, and the Sol Goldman Pancreatic Cancer Center, Johns Hopkins University, Baltimore, MD 21287, United States
Author contributions: Rucki AA wrote the manuscript; and Zheng L developed the concept and revised the manuscript.
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Correspondence to: Lei Zheng, MD, PhD, Department of Oncology, The Sidney Kimmel Cancer Center, the Skip Viragh Clinical Pancreatic Cancer Center, and the Sol Goldman Pancreatic Cancer Center, Johns Hopkins University, 1650 Orleans Street, CRB1 Room 488, Baltimore, MD 21287, United States. lzheng6@jhmi.edu
Telephone: +1-410-5026241 Fax: +1-410-4168216
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Abstract

Pancreatic ductal adenocarcinoma (PDA) is among the deadliest cancers in the United States and in the world. Late diagnosis, early metastasis and lack of effective therapy are among the reasons why only 6% of patients diagnosed with PDA survive past 5 years. Despite development of targeted therapy against other cancers, little progression has been made in the treatment of PDA. Therefore, there is an urgent need for the development of new treatments. However, in order to proceed with treatments, the complicated biology of PDA needs to be understood first. Interestingly, majority of the tumor volume is not made of malignant epithelial cells but of stroma. In recent years, it has become evident that there is an important interaction between the stromal compartment and the less prevalent malignant cells, leading to cancer progression. The stroma not only serves as a growth promoting source of signals but it is also a physical barrier to drug delivery. Understanding the tumor-stroma signaling leading to development of desmoplastic reaction and tumor progression can lead to the development of therapies to decrease stromal activity and improve drug delivery. In this review, we focus on how the current understanding of biology of the pancreatic tumor microenvironment can be translated into the development of targeted therapy.

Core tip: This is a comprehensive review and an update on recent progresses in understanding the role of tumor microenvironment in the growth, invasion and metastasis of pancreatic cancer. The role of tumor microenvironment in anti-tumor immune response and treatment of pancreatic cancer is also reviewed. How our knowledge in tumor microenvironment is translated into the development of pancreatic cancer therapy is discussed.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease. It is the 4th leading cause of cancer related deaths in the United States and according to latest statistics the incidence rate is on a rise. The high mortality rates are founded on the fact that PDA is very resistant to chemotherapy and radiation. Most patients are diagnosed at late/metastatic stages of the disease. Less than 20% of patients diagnosed with PDA are eligible for surgical resection, and out of those most present with high incidence of metastasis after resection[1]. This aggressiveness contradicts the finding that majority of the tumor volume is not composed of neoplastic cells, but consists of the stroma/desmoplastic reaction to the cancer[2,3]. In recent years, it has become evident that the desmoplastic reaction is not only a bystander, but it is a source of cellular and molecular components that promote tumor progression and metastasis[4,5]. Importantly, increased levels of stroma correlate with poor prognosis[6] and depletion of the stromal compartment has been associated with improved prognosis in both preclinical and clinical trials[7-9] making pancreatic tumor stroma a valid therapeutic target. Despite the broader understanding of PDA biology very little progress has been made in terms of treatment development. Gemcitabine was approved for PDA treatment over a decade ago, however it still remains the standard of care[10]. The recent breakthrough phase III clinical trial evaluating combination therapy FOLFIRINOX (oxaliplatin/irinotecan/5-FU/leucovorin) showed increase in overall survival by 4.3 mo when compared to gemcitabine but it also resulted in increased toxicity. Results of the study led to approval of this drug combination for patients with metastatic PDA and good performance status[11]. The development of new therapeutics for PDA has been progressing very slowly, nevertheless the devastating PDA statistics call for an urgent advance in effective treatment strategy. In this review, we will discuss the current understanding of PDA biology and how this knowledge is being translated into development of novel, targeted therapies for PDA patients.

STROMAL COMPONENTS OF PANCREATIC CANCER

The histological hallmark of PDA is the dense stroma surrounding malignant epithelial cells. The stroma, also referred to as desmoplastic reaction consist of numerous cellular as well as acellular constituents. The cellular components include fibroblasts, stellate cells, immune cells, endothelial cells, and nerve cells. The acellular compartment is comprised of extracellular matrix (ECM) (*i.e.*, collagen, fibrinogen, hyaluronan, and fibrin) as well as variety of other proteins, enzymes, and growth factors (Figure 1).

Fibroblasts

Activated fibroblasts, also referred to as pancreatic stel-

late cells (PSCs), have been given much attention in the past years. PSCs in their quiescent form are found in minimal numbers in normal, healthy pancreas[12]. Their homeostatic role is still poorly understood, however they have been shown to contain fat droplets in their cytoplasm; indicating potential role in lipid metabolism; have low mitotic index and low capability of ECM synthesis[13]. In PDA, on the other hand, PSCs become activated, as determined by their myofibroblastic phenotype and expression of alpha smooth muscle actin[14]. Activated PSCs have been shown to be a source of ECM, growth factors and immune modulatory signals[15-17]. Molecular signals originating from PSCs are conveyed to neoplastic cell promoting tumor proliferation and invasion, cancer stem cell maintenance and generation of immunosuppressive environment[13,16-21]. Similarly, neoplastic cells send stimulatory signals to PSCs providing a positive feedback loop that promotes cancer progression[22]. The population of stromal fibroblasts is very heterogeneous and numerous markers have been utilized to characterize stromal cells[23]. PSCs, which are regarded as alpha smooth muscle actin expressing cells are phenotypically similar to a broader population of fibroblasts marked by the surface glycoprotein expression of fibroblast activation protein (FAP)[14]. This similarity is based on the ability of both cell types to promote tumor proliferation and invasion, secretion of collagen types I , III, and IV, fibronectin, laminin, hyaluronan, and various growth factors[14,24]. Pro-tumorigenic properties of FAP expressing fibroblasts have made them an attractive target for PDA therapy (discussed later).

Extracellular matrix

Another component of the tumor microenvironment is the ECM. This acellular part of pancreatic tumor stroma is composed of variety of fibrous proteins (*i.e.*, collagen), polysaccharides (*i.e.*, hyaluronan) and glycoproteins (*i.e.*, fibronectin). Additionally, diversity of growth factors and other proteins are found in the ECM of PDA. This mesh of fibrous molecules not only provides support to the surrounding tissues but it also plays a role in differentiation, remodeling and homeostasis, in healthy organs[25]. Not surprisingly, different components of pancreatic stroma ECM have been shown to have tumorigenic properties. In particular, collagen I has been associated with higher expression of transgelin (gene used in this study to determine PSCs activation) when compared to other non-activating matrices[26]. In other studies, collagen I was linked to resistance to gemcitabine, a standard cytotoxic drug used for pancreatic cancer treatment[27,28]. Hyaluronan (HA), a non-typical glycosaminoglycan with high capacity of water retention, has recently become an attractive target for pancreatic cancer therapy. HA is expressed in high levels in PDA and its abundance has been connected to increased intratumoral fluid pressure and consequent vascular collapse[29,30]. PDA, unlike many solid tumors is hypovascular, moreover, the blood vessels that are present in the intratumoral space have been reported to be mostly nonfunctional[30]. The lim-
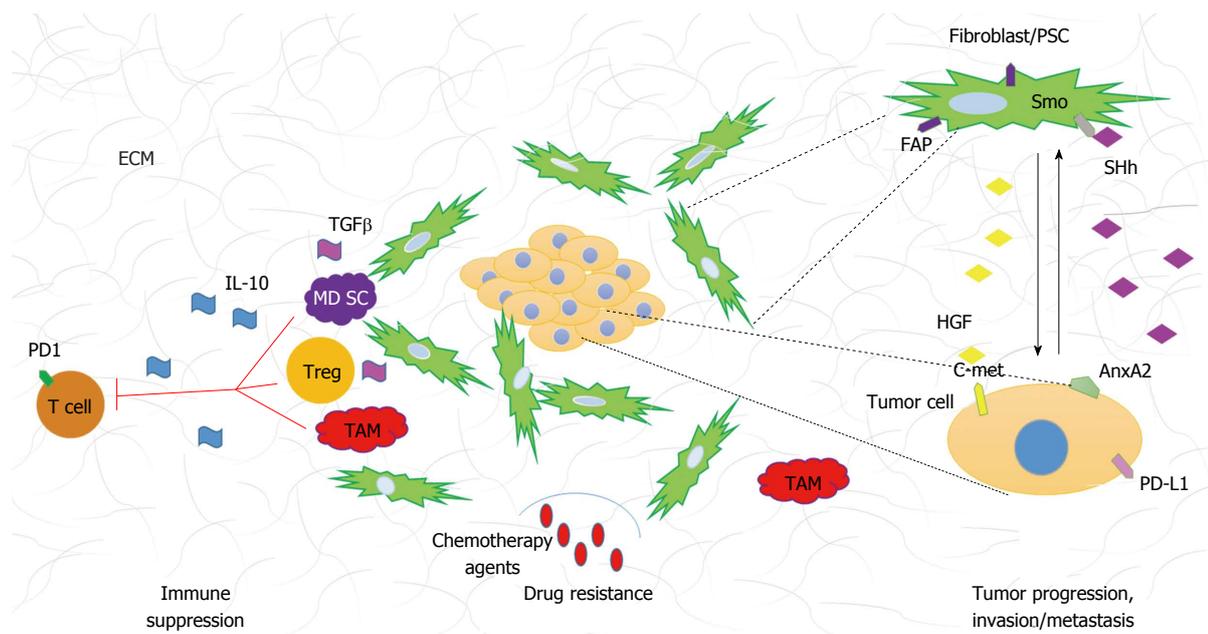
Figure 1 Graphical representation of the stromal components and their interactions in pancreatic ductal adenocarcinoma. FAP: Fibroblast activation protein; Smo: Smoothened; SHh: Sonic hedgehog; HGF: Hepatocyte growth factor; PD-L1: Programmed death ligand-1; PD1: Programmed death-1 (receptor); AnxA2: AnnexinA2; TGFβ: Transforming growth factor β; IL-10: Interleukin 10; MDSC: Myeloid derived suppressor cell; Treg: T regulatory cell; TAM: Tumor associated macrophage.

ited numbers of functional blood vessels in PDA and the dense stroma are believed to be among the reasons why intravenous chemotherapeutic agents as well as the recently tested antiangiogenic drugs[31] do not elicit great effect on the tumor cells.

Immune cells

Broad repertoire of immune cells including both adaptive and innate cell types are also present in the PDA tumor microenvironment. Tumor infiltrating immune cells have been implicated in tumor progression, chemotherapy resistance and metastasis[32-34]. Additionally, the immune infiltration is evident in early premalignant lesions and increases with PDA progression[32]. Immune suppression and immune tolerance to tumor associated antigens is one of the characteristics of PDA and it is associated with poor prognosis[33,35]. The abundance of suppressive cells leads to low numbers of effector CD8+ T cells in the PDA stroma and consequently limited anti-tumor cytotoxicity[32]. Among the most plentiful tumor infiltrating immune cells characterized by their suppressive phenotype are myeloid derived suppressive cells (MD-SCs), T regulatory cells (Tregs), and tumor associated macrophages (TAMs). The suppressive cell population is characterized by its ability to prevent activation and functionality of effector cells leading to diminished tumor cytotoxicity[36]. The immune modulatory cell population regulates effector cells anti-tumor responses by variety of mechanisms. MDSC inhibit CD8+ T cell function *via* arginase, and reactive oxygen species secretion, which requires direct cell contact[37,38]. Tregs ability to decrease effectors function is partially due to their ability to secrete suppressive cytokines such as interleukin 10 (IL-10) and tumor growth factor β (TGFβ) but they can also

be cell contact dependent where proteins like CTLA-4 and PD-1 are involved[39]. TAMs can be divided into two functional subtypes: M1 (pro-inflammatory) and M2 (immunosuppressive). The M2 subtype cells are a source of anti-inflammatory cytokines such as IL-10 and have been shown to induce Th2 responses (also found to be immunosuppressive in PDA)[40]. In addition to the presence of immunosuppressive cell populations in the PDA microenvironment, the numbers of effector cells such as CD4+, CD8+ T cells and NK cells are minimal. More importantly, infiltrating CD8+ and CD4+ T cells have either naïve phenotype or are nonfunctional, antigen experienced effectors[32]. There are numerous mechanisms that have been implicated in the non-functionality of antigen experienced T cells. Checkpoints and inhibitory receptors like PD-1 are examples of proteins that transduce inhibitory signals during lymphocyte activation[41]. Tumor cells can also express ligands such as the PD-L1 protein that have been shown to dampen immune anti-tumor responses. Upregulation of those inhibitory molecules, PD-L1 in particular, has been associated with poor prognosis[42,43]. Lastly, TGFβ has been shown to play a role in Th17 subtype differentiation[44]. Interestingly, TGFβ dependent differentiation of Th17 cells has been implicated with increased immunosuppressive abilities[45]. Both tumor cells and cancer associated fibroblast secrete TGFβ and increased levels of IL-17 secreting CD4+ cells (Th17) have been found in PDA tumor microenvironment[46]. To date, the role of Th17 subtype of immune cells remains controversial in cancer biology, as it has been shown to have both pro- and anti-tumorigenic properties[47-49]. It is important to mention, that the role of Th17 cells is well documented in promoting fibrosis[50,51]. Hepatic stellate cells (HSCs) have been shown to become activated in

response to Th17 secreted factors[50,52], and because PSCs resemble HSCs, it would be interesting to investigate the role of Th17 immune subtype on PSCs activation and desmoplastic reaction.

Subsequently, modulation of the pro-tumorigenic immune infiltration by either depletion of suppressive cells, polarization of the cell population to more anti-tumor phenotype, checkpoint blockade or increase of activity of the effector cells can be exploited in cancer therapy.

SIGNALING NETWORKS IN THE TUMOR MICROENVIRONMENT

Tumor-stroma interactions create a very complicated signaling network that drives tumor progression. Many signaling pathways have been associated with PDA tumorigenesis, in this review we will focus on paracrine pathways that originate in the neoplasm and contribute to the development of desmoplastic reaction (Figure 1).

Sonic hedgehog

Sonic hedgehog (SHh) is a developmental signaling pathway that is crucial for organ development during embryogenesis. Briefly, in the absence of ligand (SHh) the signaling pathway is inactive and the cell surface receptor Patched (Ptch) inhibits translocation of smoothened (Smo) to the cell surface. Upon ligand binding, Ptch relives the repression on Smo allowing it to translocate to cell surface. The translocation of Smo is a key activating step in downstream signaling. Gli1/2/3, which belong to the zinc-finger transcription factor family are the downstream effectors of Smo activation. Ligand binding to Ptch, results in the translocation of Gli1 (activator) to the nucleus allowing expression of SHh associated genes. During activation of the pathway, Gli2/3 (repressors) are nonfunctional. In the absence of ligand binding, Gli2/3, undergo proteolytic cleavage, move to nucleus and repress transcription of SHh dependent genes. In the inactive state, Gli1 is rendered nonfunctional[53]. SHh is overexpressed by PDA tumor cells, however its function is restricted to the stromal compartment forming a paracrine signaling network that promotes and maintains desmoplasia[54-56]. It has been also noted that only cancer associated fibroblasts and not the neoplastic cells show SHh pathway activation and Smo receptor overexpression[57]. Importantly, Olive *et al*[7] demonstrated that the use of a SHh inhibitor in preclinical mouse model of pancreatic cancer, resulted in better delivery of gemcitabine through reduction of stroma and increase of vascular density. It is important to mention that even though cancer associated fibroblasts (CAFs) are an established target of SHh pathway activation, recent study of pancreatic cancer stem cells (cancer initiating cells) showed that Smo is overexpressed in this population of tumor cells. Pancreatic cancer stem cells alike CAFs have been shown to be susceptible to SHh inhibition and should be considered a target[58].

TGFβ

The notion that depletion of stromal compartment allows for better drug delivery in PDA brought upon re-examination of another signaling pathway that has been linked to regulation of desmoplastic reaction, the TGFβ signaling pathway. This signaling cascade involves three TGFβ ligands and three receptors. In short, binding of ligand to its receptor (type Ⅱ) results in recruitment and phosphorylation of type Ⅰ receptor and downstream propagation of molecular signals. The effector molecules in this cascade are the proteins of SMAD family, which upon phosphorylation, dimerize, translocate to the nucleus and regulate expression of TGFβ associated genes[59]. TGFβ is overexpressed in PDA and its overexpression correlates with poor survival[60]. TGFβ's involvement in pancreatic cancer is complicated as it has been shown to affect both the stromal and the neoplastic compartments. Elevated levels of TGFβ have been shown to impact cell proliferation, immunosuppression and activation of PSCs[61-64]. In mouse models, overexpression of SMAD 7 (TGFβ inactivator) showed decreased ECM production, less fibrosis and more importantly diminished PSCs activation[65]. Additionally, TGFβ has been demonstrated to drive epithelial to mesenchymal transition (EMT) process, believed to be the initial step of metastasis[66]. EMT was first characterized in development, in which the process is vital for embryogenesis and organogenesis. The cellular characteristics of EMT include the loss of epithelial cells polarity, cell adhesion, gain of mobility and invasive properties resulting in phenotypical changes that resemble mesenchymal cells[67]. On a molecular level, EMT is described by the changes in gene/protein expression that occur in this process. Specifically, upregulation of mesenchymal markers (vimentin, fibronectin, N-cadherin, Snail and Slug) and downregulation of epithelial markers (E-cadherin, zonula-occludens and nuclear translocation of β-catenin) are routinely used to determine the presence of EMT[67,68]. In recent years, many different pathways have been implicated in the initiation of EMT and consequent cancer invasion and metastasis, of which TGFβ is an example[66,69-74]. EMT has also been linked to induction and maintenance of cancer stem cell population in PDA[75]. Importantly, the presence of EMT markers (as discussed above) has been shown to correlate with higher lymph node metastasis and decreased survival in PDA patients[76]. Taken together, the pleotropic functionality of TGFβ in cancer makes it a valid target for patients with PDA.

Others

There are many other signaling pathways that have been associated with PDA development, progression and metastasis. We will discuss them briefly. Expression of Delta-like ligand 4, a protein involved in the developmental Notch pathway has been linked to worst prognosis in patients who underwent surgical resection of pancreatic tumor[77]. Moreover, inhibition of γ-secretase, a protein

that allows Notch signaling propagation to take place and that is often constitutively active in PDA, showed regression of primary tumors, reduced metastasis and decrease of pancreatic stem cell population when combined with gemcitabine[78]. Another pathway that recently gained attention is the c-met pathway. It is well documented that c-met receptor and its ligand HGF are upregulated in PDA. C-met and HGF are detected early in PDA development but are not sufficient to promote tumorigenesis without other oncogenic changes[79]. Recently the expression of c-met has been linked to the stem cell population and because HGF is exclusively secreted by stromal fibroblasts, paracrine relationship between the stroma and neoplasm to promote cancer progression has been suggested. Importantly, studies with c-met inhibitors showed increase of apoptosis and sensitivity to gemcitabine in malignant cells[80]. Moreover, stromal expression of HGF was correlated with decreased disease free survival[81] proposing that HGF/c-met targeting can be beneficial to patients with PDA. Another attractive target is the annexinA2 pathway shown to play an important role in pancreatic tumor metastasis and EMT. Inhibition of tyrosine 23 phosphorylation of annexinA2 was shown to reduce invasion *in vitro,* and metastasis *in vivo.* Although, the kinases responsible for annexinA2 phosphorylation in PDA remain to be confirmed, IGF-1R and Src have been proposed to be involved[74].

Nuclear factor kappa-B (NF-κB), a transcription factor, regulates genes involved in inflammation, cell proliferation and survival[82]. NF-κB signaling pathway activity has been documented to be upregulated in PDA but not in normal pancreatic tissue[83,84]. Activation of this signaling cascade has also been linked to early processes in PDA development. Liou *et al*[85], recently reported that macrophage secreted cytokines initiate acinar to ductal metaplasia *via* activation of NF-κB and consequent upregulation of matrix metalloprotinases (MMPs). To date, direct targeting of NF-κB has been shown to be challenging[86,87]. As an alternative to the direct NF-κB inhibition, upstream activators and downstream effectors of the signaling pathway should be evaluated.

Numerous signaling pathways have been explored as potential targets for pancreatic cancer therapy, however review of all of them goes beyond the scope of this article.

TARGETING OF STROMAL COMPARTMENTS AND CLINICAL APPLICATIONS

Understanding complex stromal constituents and involvement of numerous signaling pathways in PDA progression and desmoplastic reaction is crucial to the development of novel therapies. It has become evident that targeting the stromal components has undeniable benefit for preclinical mouse models of PDA. However, translating those findings to the patients care can be challenging. There are numerous ongoing clinical trials utilizing the above described targets that show encouraging results. In this part of the review we will briefly discuss the most promising ones (Table 1).

SHh
SHh pathway inhibition shows beneficial effect in patients with other cancers such as basal cell carcinoma for which Vismodegib (GDC-0449) has been FDA approved in 2012[88]. Inhibition of SHh in preclinical mouse models showed better gemcitabine delivery, stromal depletion and increased vascularization of PDA tumors[7]. Thus, different SHh inhibitors have recently been tested in clinical trials in combination with gemcitabine or FOLFIRINOX for metastatic PDAs[89]. Additionally, GDC-0449 is now being tested in combination with *nab*-paclitaxel (human-albumin-bound paclitaxel, Abraxane) and gemcitabine in phase II clinical trial in patients with previously untreated metastatic PDA (clinical trial # NCT01088815) to evaluate disease free survival and toxicity. Although IPI-926 (Smo inhibitor) given in combination with gemcitabine showed partial responses in 3 out of 9 patients, the combination of IPI-926 and gemcitabine did not yield any survival benefit comparing to gemcitabine alone[90]. Therefore, targeting the stroma of PDA through SHh inhibition and simultaneous modulation of other stromal signaling should be explored.

Hyaluronidase
Another stromal target showing encouraging results in phase Ib clinical trials is hyaluronan. As shown in mouse models of PDA, enzymatic degradation of hyaluronan resulted in increased gemcitabine tumor cytotoxicity due to relief of vascular collapse[30]. Those prove of principle experiments lead to the development of PEGPH20 (pegylated recombinant human hyaluronidase- an enzyme that degrades hyaluronan). Administration of PEGPH20 to PDA patients with advanced disease (stage IV) in combination with gemcitabine revealed partial response in 43% of patients and stable disease in additional 30% patients in phase I b clinical trials. More impressively, the partial response rate was 64% in those patients whose PDAs expressed high level of hyaluronan[91]. This high response rate has led to further testing of PEGPH20 in combination with gemcitabine and *nab*-paclitaxel in a randomized phase II clinical trial.

TGFβ
Trabedersen, a type II TGFβ antisense inhibitor is also being tested in clinical trials in patients with advanced pancreatic adenocarcinoma and malignant melanoma. Although phase II clinical trial results have not been released yet, phase I reports revealed that trabedersen was well tolerated and showed median overall survival to be 13.2 mo. In addition, one patient presented with a stable disease after 14.8 mo of last treatment[92].

Immune system modulation/activation
Immunotherapy approaches are being tested in clinical

Table 1 Recent and ongoing preclinical and clinical studies of experimental therapies targeting tumor microenvironment of pancreatic ductal adenocarcinoma

Stromal component	Therapeutic target	Treatments in preclinical and clinical trials	Up to date preclinical/clinical trial results
PSCs/fibroblasts	FAP	Sibrotuzumab (colorectal cancer)	Hofheinz *et al*[99], 2003
ECM	Hyaluronan	PEGPH20	Strimpakos *et al*[91], 2013
	MMPs	BAY 12-9566	Moore *et al*[100], 2003
		Marimastat	Bramhall *et al*[101], 2002
Immune cells	PD-L1	BMS-936559	Brahmer *et al*[102], 2012
	CTLA-4	Ipilimumab	Le *et al*[95], 2013
	CD8+ T cells	GVAX	Lutz *et al*[93], 2011
			Laheru *et al*[94], 2008
	CD40	CP-870,893	Beatty *et al*[103], 2013
Signaling pathways mediating tumor-stroma interactions	Smo/SHh	Vismodegib (GDC-0449)	Stephenson *et al*[90], 2011
		IPI-926	
	Type Ⅱ TGFβ receptor	Trabedersen	Oettle *et al*[92], 2009
	γ-secretase (Notch pathway)	PF-03084014 (preclinical)	Yabuuchi *et al*[78], 2013 (preclinical)
	HGF/c-met	Many different compounds (solid cancers)	Venepalli *et al*[104], 2013 (solid cancers)
	Different molecules in NF-κB cascade	Many different compounds (*i.e.*, curcumin, proteasome inhibitor)	Arlt *et al*[105], 2012

ECM: Extracellular matrix; MMP: Matrix metalloproteinase; PD-L1: Programmed death receptor ligand 1; CTLA-4: Cytotoxic T-lymphocyte antigen 4; SHh: Sonic hedgehog; Smo: Smoothened; TGFβ: Transforming growth factor β; HGF: Hepatocyte growth factor; NF-κB: Nuclear factor κ-B; PSC: Pancreatic stellate cell; FAP: Fibroblast activation protein.

trials for PDA with a goal to induce tumor infiltration and activation of effector cells (*i.e.*, CD8+ T cells) and consequent CD8+ T cell dependent tumor lysis.

Multiple clinical trials of a lethally irradiated allogeneic GM-CSF secreting whole cell vaccine (GVAX) administered to patients with resected PDA or metastatic PDA demonstrated that enhanced response of interferon-γ secreting mesothelin-specific CD8+ T cells in peripheral lymphocytes correlates with better survival[93,94]. A pilot study testing the combination of GVAX and ipilimumab (an anti-CTLA-4 therapeutic antibody) comparing to ipilimumab alone showed a trend of increase in overall survival in metastatic PDA patients that have been previously treated with multiple lines of chemotherapy and thus supported the role of CTLA-4 blockade in enhancing anti-tumor response of GVAX[95]. However, it remains to be explored how vaccine-based immunotherapy activates anti-tumor effector cells within tumor microenvironment. Identification of new targets in tumor microenvironment may enhance the development of immune modulatory therapies.

A potential immune modulatory target in tumor microenvironment is CD40. CD40 is a costimulatory molecule found on antigen presenting cells (APCs) that is required for their activation by CD4+ helper cells. Only activated APCs can in turn activate naïve CD8+ T cells into cytotoxic effector cells. Key studies showed that using CD40 activating antibody can effectively stimulate APCs in the absence of CD4+ helper cells, which then can successfully prime and activate CD8+ T cells[96]. Those preclinical studies led to development of activating CD40 antibodies, which have been tested in clinical trials. One study showed that combination of CD40 agonist with gemcitabine resulted in tumor regression in patients not eli-

gible for tumor resection. Interestingly, it was noted that the tumoricidal cells were CD40 activated macrophages and not CD8+ T cells as originally expected. The treatment with CD40 agonist resulted in stroma depletion and increased numbers of tumor infiltrating activated macrophages[8].

How stromal fibroblast cells can modulate anti-tumor immune response has been investigated in preclinical studies. One study demonstrated that depletion of fibroblast activation protein-α (FAP)-expressing stromal cells in PDA resulted in an immune-mediated hypoxic necrosis of both tumor and stroma cells[97]. Additionally, targeting of cancer stroma fibroblasts with FAP-activated promelittin protoxin, showed increased tumor lysis and growth inhibition in xenograft mouse models of breast and prostate cancer[98]. However, targeting FAP positive stromal cells with humanized anti-FAP antibodies tested in phase Ⅱ clinical trials in patients with metastatic colorectal cancer did not report encouraging results[99]. Taking into consideration the outcomes from both preclinical and clinical studies, it is reasonable to propose that FAP-targeted stromal depletion shows immune activating effect, but requires additional immune modulation to be effective. It is plausible that simultaneous FAP-targeted stromal depletion and immune activation, by either vaccination or immune checkpoint blockade would result in increased benefits for PDA patients.

CONCLUSION

Despite the broad number of clinical trials, there is still a lack of groundbreaking therapies for patients affected by pancreatic cancer. Thus, targeting only the neoplastic cells has not resulted in a substantially improved PDA

treatment. It is now well established that the desmoplastic reaction present in PDA is not just a bystander but it is a source of different cellular and acellular factors that promote tumor progression, immunosuppression and metastasis. Targeted therapies to deplete stromal compartments have shown improved chemotherapy delivery and reduction of immunosuppression in preclinical models. There is still much work to be done in order to decipher the complicated interactions between stroma and neoplastic cells in PDA. It is clear, however, that future studies should not be limited to one component of PDA. Application of targeted therapy to deplete the tumorigenic stromal compartment along with inhibition of cancer promoting signaling pathways should be evaluated. Moreover, future studies ought to test the combination of agents that target the stroma and those that activate anti-tumor immune responses. Treatments that can reduce desmoplastic reaction, overcome immune suppression and inhibit tumorigenic signaling pathways may lead to more successful patient care.

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2014 ADVANCES IN PANCREATIC CANCER

Embryonic stem cell factors and pancreatic cancer

Marta Herreros-Villanueva, Luis Bujanda, Daniel D Billadeau, Jin-San Zhang

Marta Herreros-Villanueva, Daniel D Billadeau, Jin-San Zhang, Division of Oncology Research, Schulze Center for Novel Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, United States

Marta Herreros-Villanueva, Luis Bujanda, Department of Gastroenterology, Hospital Donostia/Instituto Biodonostia, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Universidad del País Vasco UPV/EHU, 20014 San Sebastián, Spain

Jin-San Zhang, School of Pharmaceutical Sciences, Key Laboratory of Biotechnology and Pharmaceutical Engineering, Wenzhou Medical University, Wenzhou 325000, Zhejiang Province, China

Correspondence to: Jin-San Zhang, MD, PhD, Division of Oncology Research, Schulze Center for Novel Therapeutics, Mayo Clinic College of Medicine, Rochester, MN 55905, United States. zhang.jinsan@mayo.edu
Telephone: +1-507-2669310   Fax: +1-507-2665146

Abstract

Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic tumor, is a highly aggressive human cancer with the lowest five-year survival rate of any human maligancy primarily due to its early-metastasis and lack of response to chemotherapy and radiation. Recent research suggests that PDAC cells comprise a hierarchy of tumor cells that develop around a population of cancer stem cells (CSCs), a small and distinct population of cancer cells that mediates tumoregenesis, metastasis and resistance to standard treatments. Thus, CSCs could be a target for more effective treatment options. Interestingly, pancreatic CSCs are subject to regulation by some of key embryonic stem cell (ESC) transctiption factors abberrently expressed in PDAC, such as SOX2, OCT4 and NANOG. ESC transcription factors are important DNA-binding proteins present in both embryonic and adult somatic cells. The critical role of these factors in reprogramming processes makes them essential not only for embryonic development but also tumorigenesis. Here we provide an overview of stem cell transcription factors, particularly SOX2, OCT4, and NANOG, on their expression and function in pancreatic cancer. In contrast to embryonic stem cells, in which OCT4 and SOX2 are tightly regulated and physically interact to regulate a wide spectrum of target genes, *de novo* SOX2 expression alone in pancreatic cancer cells is sufficient to promote self-renewal, de-differentiation and imparting stemness characteristics *via* impacting specific cell cycle regulatory genes and epithelial-mesnechymal transtion driver genes. Thus, targeting ESC factors, particularly SOX2, could be a worthy strategy for pancreatic cancer therapy.

Core tip: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancer due to its early metastasis and lack of response to chemoradiotherapy. Pancreatic cancer stem cells (CSCs) are implicated in tumorigenesis and metastasis as well as therapy resistance, therefore represent a potential target for effective therapeutic options. Recent publications including our own research demonstrate that key embryonic stem cell (ESC) factors, such as OCT4, NANOG and SOX2, are abbrently expressed in PDAC and contribute to pancreatic CSC-like characteristics, such as self-renewal and de-differentiation. This review aims to summarize our current knowledge on the role of ESC

factors particulary SOX2 in regulating pancreatic CSC-like feature and implication for therapy.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in men and women in the United States. In 2012 alone, an estimated 43920 adults in the United States were diagnosed with pancreatic cancer and 37390 deaths from this disease occurred[1]. About 280000 new cases of pancreatic cancer were recorded in 2008 worldwide. Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer, is also the most lethal among the human solid tumors with a 5-year survival rate of less than 5 percent[2]. The main reasons for this outcome include lack of early detection, invasive behavior and intrinsic resistance to most chemo-/radio- and immuno-therapy strategies[3,4]. Recently, several studies have identified PDAC cancer stem cells (CSCs), which are highly tumorigenic and have the capacity to not only self-renew, but also generate differentiated progeny[3,5-7]. Pancreatic CSCs are also resistant to chemotherapies commonly used to treat patients with PDAC[8-10]. Thus, studies identifying key determinants in pancreatic cancer and pancreatic CSCs can provide both biomarkers of PDAC aggressiveness and potentially optimal targets to overcome chemoresistance (Table 1). Here we review how embryonic stem factors contributes to the agressiveness of this disease and the potential for targeted therapy.

STEM CELL DEFINITION AND TYPES

Stem cells (SCs) are traditionally defined as cells that can both self-renew and generate a progeny that are capable of following more than a single differentiation pathway[11]. Currently, four types of SCs have been described[12]. The first two are physiologically present at different stages of life, namely, the embryonic stem cells (ESCs) and the somatic or adult stem cells (ASCs). The ESCs are the best studied SCs and knowledge derived from ESCs research has guided the investigations of other types of SCs. ASCs are postnatal derivatives of ESCs located throughout the body. ASCs have been shown to retain co-expression of at least three of the core transcription factors characteristic of ESCs (OCT4, KLF4, and SOX2). Similar to ESCs, the presence of a balanced network of core stem markers, rather than the overt expression of a single factor, contributes to maintenenance of ASC characteristics. The third SC type is induced

pluripotent stem cells (iPSCs), which are artificially engineered from a non-pluripotent cell, such as *via* somatic cell nuclear transfer or reprograming with gene transfer. The generation of iPSCs represents a milestone achievement in SC research, which not only breaks the dogma that somatic cell differentiation is an irreversible process, but also makes possible a new approach for regenerative medicine without controversial use of embryos. The fourth SC type is CSCs, also referred to as cancer initiating cells (CICs), which are defined as those cells within a tumor that can self-renew, produce differentiated progeny, and drive tumorigenesis. The ability of cancer cells to form nonadherent spheroids *in vitro* culture is frequently used as a surrogate of stemness. Unlike ESCs, CSCs are highly heterogenous with great variation among the markers for each tumor type.

ESCs AND ESC TRANSCRIPTION FACTORS

ESCs are derived from the inner cell mass (ICM) of the preimplantation mammalian embryo and can be maintained indefinitely in culture[13]. By definition, ESCs are pluripotent. They are able to give rise to all somatic and the three germ cell lineages of the developing embryo. Pluripotency is maintained through self-renewal, which allows ESCs to duplicate themselves without losing the ability to differentiate. This can be achieved *via* both symmetric and asymmetric cell divisions[14].

Over the last decade, there has been accumulating evidence indicating that the maintenance of pluripotency in ESCs is governed by core genetic and epigenetic regulators, which allow self-renewal and prevents specific differentiation pathways. Recent progress on the molecular mechanism(s) governing stem cells pluripotency has provided critical insights into the role of nine core transcription factors OCT4 (POU5F1), NANOG, SOX2, Dppa4, Dppa5, Sall4, Utf1, Rex2, and Rif1 in maintaining mouse cells in the undifferentiated stage[15-18]. Among these genes, OCT4, NANOG, and SOX2, referred to as pluripotency genes, are highly expressed in the ICM. The perfect balance of these proteins maintains pluripotency and self-renew in ESC during the first days of embryonic development[18]. Broadly, the pluripotency genes have been shown to be common to all SC types (Figure 1). In contrast to *OCT4*, *NANOG* and *SOX2*, *c-MYC*, an important oncogene as well as a reprogramming factor for pluripotency[17], is highly heterogeneous in cells from the ICM. However, it is not always considered a pluripotency gene in ESCs. The activity of these three core pluripotency genes regulates and coordinates the expression of a second set of core genes, which include transcription factos, cell surface markers, ABC transporters, and enzymes. Together, these proteins orchestrate the specific stem cells properties[19].

SOX2 and OCT4 form a protein complex in the nucleus of ESCs. This complex is auto-regulated in a loop

Table 1	Biological role and clinical implications for embryonic stem cell factors in pancreatic cancer		
Gene	**Biological role/behaviour**	**PDAC implications**	**Ref.**
OCT4	Overexpressed in 69% of PDAC. Pro-oncogenic role	Correlation with N1/M1 status and indicative of worse prognosis	Polvani *et al*[47], 2013
	Overexpressed in 48.8% of PDAC. Induces cell proliferation, migration and invasion	Contribution to metastasis and drug resistance	Lu *et al*[50], 2013
	Overexpressed in human cell lines	Multidrug resistance and metastasis	Wang *et al*[52], 2013
	Induction of tumorigenic capacity	Chemo-resistance	Wang *et al*[53], 2013
	Overexpressed in 79.2% metaplastic ducts	Early carcinogenesis and worse prognosis	Wen *et al*[49], 2010
SOX2	Overexpressed in poorly differentiated human tumors	Correlation to aggressiveness	Sanada *et al*[54], 2006
	Ectopic expression in 19.3% of PDAC. Promotes cancer cell proliferation/dedifferentiation	Rapid tumor progression and poor differentiation	Herreros-Villanueva *et al*[38], 2013
	Induction of tumorigenic capacity	Chemo-resistance	Wang *et al*[53], 2013
NANOG	Overexpressed in 53.5% of PDAC. Induces proliferation, migration and invasion	Associated with eraly stage carcinogenesis and worse overall survival	Lu *et al*[50], 2013
	Overexpressed in cells capable of initiating spheres	Resistance to 5-FU treatment	Lonardo *et al*[68], 2013
	Overexpressed in pancreatic tumors	Contribution to carcinogenesis and correlates to worse prognosis	Wen *et al*[49], 2010

PDAC: Pancreatic ductal adenocarcinoma.
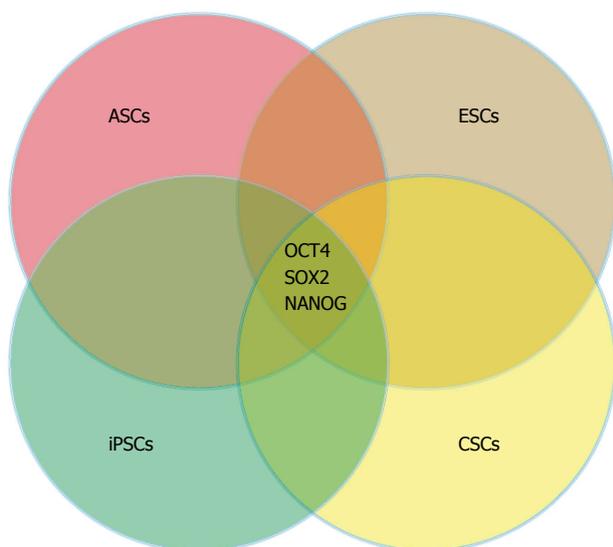
Figure 1 Overlapping expression of SOX2, NANOG, and OCT4 in all four types of stem cells: embryonic stem cells, adult stem cells, induced pluripotent stem cells, and cancer stem cells. ASCs: Adult stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; CSCs: Cancer stem cells.

that, transcriptionally, also induces the expresion of pluripotency genes (most importantly NANOG), cell cycle, apoptosis, DNA repair, chromatin structure genes, and genes regulating endoderm, mesoderm, and ectoderm differentiation. Thus, tight control of all these genes may allow ICM cells to exit from their inherent developmental program, as they acquire the ability to self-renew, while retaining pluripotency as ESCs[20]. Finally, when the expression of these pluripotency genes decreases in a properly regulated way, an induction in the expression of early differentiation markers occurs. These markers include ectoderm markers (*Pax6*, *Otx1*, *Neurod1*, *Nes*, *Lhx5*, and *Hoxb1*), mesoderm markers (*Tbx2*, *T*, *Nkx2-5*, *Myod1*, *Myf5*, *Mesdc1*, *Mesdc2*, *Kdr*, *Isl1*, *Hand1* and *Eomes*), endoderm markers (*Onecut1*, *Gata4*, *Gata5*, and *Gata6*),

and extraembryonic markers (*Cdx2* and *Tpbpa*).

KEY ESC FACTORS IN IPSCs

iPSCs were first derived by the transduction of mouse and human fibroblasts through integrating viruses carrying four transcription factors: OCT4, SOX2, MYC and Krupple-like factor 4 (KLF4)[21], also referred as the Yamanaka factors. Takhashi and Yamanaka[21] broke a dogma in developmental biology by showing that mammalian somatic cell differentiation is a reversible process[17,21]. By transfecting human somatic cells with the four Yamanaka factors, they were able to revert the differentiated cells to an embryonic-like state. Because these newly generated cells showed the morphology, pluripotency, and capacity to form teratomas similar to ESCs, they named these cells iPSCs. Later, Yu *et al*[22] further demonstrated that the combination of OCT4, NANOG, SOX2 and Lin28, also called Thomson Factors, was able to produce iPSCs. Both, Yamanaka and Thomson Factors are Reprogramming Factors as Reprogramming is the process that converts differentiated cells back to pluripotent cells, namely the reversal of differentiation.

Recently, new methods have been developed to reprogram human somatic cells with or without MYC[22,23] and to combine only some of the reprogramming transcription factors with chemical inhibitors[24-26]. However, the fact remains that OCT4, SOX2, MYC, and KLF4 reside at the heart of the reprogramming process. Given that the transcription factors in this network not only associate with one another, but also associate with many of the same proteins in the network, there is a high degree of interdependence between these transcription factors. Thus, it is not surprising that the levels of SOX2 and OCT4 need to be controlled carefully for optimal production of iPSC, or that small changes in the levels of these master regulators can lead to dramatically altered cell fates. However, it remains to be determined how
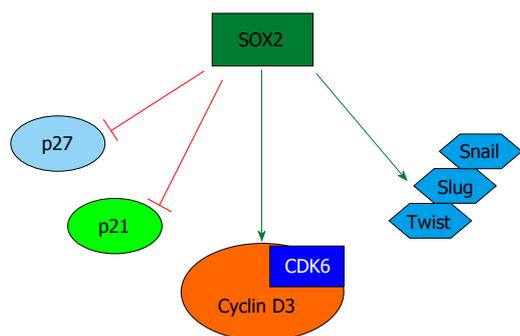
Figure 2 Diagram depicting the molecular mechanism underlying SOX2 expression-induced self-renewal and pluripotency in pancreatic cancer stem cells.

their levels affect the molecular efficiency of reprogramming. Given the strict requirement for SOX2 and OCT4 during development, their key roles in ESC differentiation, and the pronounced differences in reprogramming when their levels are not optimized, additional efforts should be made to determine why small changes in the levels of these two master regulators alters the behavior of pluripotent stem cells.

ESC FACTORS AND CSCs

Although initially discovered in hematopoietic malignancies, such as acute myelogenous leukemia and chronic myelogenous leukemia[27,28], CSCs were later described in various solid tumors, including glioblastoma[29], melanom[30], prostate[31], colon[32] head and neck squamous cell carcinoma[33], breast[34], ovarian[35], bladder[36], lung[37] and pancreatic cancer[6,7,38,39]. In these malignancies, a small population of CSCs can self-renew and differentiate into all of the other cell types forming the bulk tumoral population. However, the bulk of tumor cells lack the ability to differentiate into other subpopulations of cancer cells and thus possess limited self-renewal capacity. In addition, it has been shown that CSCs have tumor initiation capacity, forming xenograph tumors in mice and are radio- and chemo-resistant, contributing to lack of therapeutic response in patients[39].

Although several proteins have been proposed as CSC markers, there is great variation between tumor types[40,41]. This variation might be the result of the lack of standardized techniques to obtain and analyze CSCs, as well as the intrinsic plasticity of these cells[40]. Since CSCs express many genes in common with early ESCs, primarily OCT4, NANOG, and SOX2, the picture that emerged was that these transcription factors could also work together as part of a highly integrated network to regulate pluripotency and self-renewal in tumors. Nevertheless, the heterogeneity of tumors and the plasticity that characterize CSCs render the expression pattern of these transcription factors highly heterogeneous in different tumors and even within the same tumor.

Several publications show that overexpression of OCT4, SOX2 and NANOG, together or separately, led

to tumor transformation, tumorigenicity, tumor metastasis, and even distant recurrence after chemoradiotherapy[42]. It is well known that these transcription factors are more frequently overexpressed in poorly differentiated tumors (compared to well differentiated tumors) and, in theory, that the expression level of the pluripotent transcription factors should decrease with the differentiation of cells[43]. In this regard, how these genes contribute to specific CSC properties has not been fully elucidated. Based on data obtained from iPSCs, several mechanisms have been proposed to explain the properties that these transcription factors could be imparting on CSCs. For example, once these transcription factors are overexpressed, they might activate several genes whose promoters are accessible to them. These "first responders" must then engage the epigenetic machinery to remodel the chromatin through histone modification and DNA methylation. In this process, genes critical for pluripotency must be switched on, while genes responsible for differentiation must be turned off and kept off[44]. From this data, it is clear that OCT4, NANOG and SOX2 are master regulators, which together drive the transition from a somatic cell to either a CSC or iPSC (Figure 2).

ESC FACTORS AND PANCREATIC CANCER

As mentioned above, CSCs have also been described in PDAC (Table 1). Originally Li *et al*[6] identified human pancreatic CSCs as CD44+/CD24+/ESA+. A few months later, Hermann *et al*[7] showed that CD133 and CXCR4 are also expressed in cells with CSC properties. In addition, some other markers such as c-Met[5] and aldehyde dehydrogenase 1 activity (ALDH1)[45] have been demonstrated in pancreatic CSCs. Recently, some reports describe the presence of a side population (SP) of cells in pancreatic cancer, a chemoresistant population of cells that could be enriched in CSCs. Additionally, this data indicates that SP cells express pancreatic CSC markers (*CXCR4*, *CD133*) and multidrug resistance genes (*ABCB1*), associating these cells with candidate therapeutic targets and potential prognostic value[46].

The regulation and characterization of CSCs in various types of human cancer, in which SOX2, OCT4 and NANOG are important players, is currently a hot topic. However, the number of specific publications analyzing their role in pancreatic cancer is very limited. In particular, a literature search on PUBMED database using the terms "OCT4", "NANOG" and "SOX2" together with "pancreatic cancer", showed 24, 27, and 20 published articles, respectively. Furthermore, only a few of these articles discuss these factors in the context of CSCs (Table 1). Polvani *et al*[47] found that OCT4 is expressed in 69% of PDAC and that this expression correlates with N1/M1 status and clinical stage, being an independent prognostic factor for worst outcomes. In agreement with several breast cancer publications[48], patients with OCT4+ PDAC have a shorter survival, suggesting this ESC factor

as a marker of poor prognosis. Importantly, high levels of OCT4 and NANOG in human pancreatic cancer tissues were found to be associated with early stages of carcinogenesis[49] and correlate with worse prognosis[50]. Additionally OCT4 seems to contribute to multidrug-resistance and metastasis[51,52]. Wang *et al*[53] recently demonstrated that SP cells positive for NANOG, OCT4 and SOX2 possessed aggressive growth, invasion, migration and drug-resistance properties.

To date, very little is known regarding how OCT4 and NANOG contribute to pancreatic CSC properties at the molecular level. Interestingly, recent studies suggest that SOX2 is aberrantly expressed in a significant fraction of pancreatic tumors. Initially, Sanada *et al*[54] analyzed 14 cases of human PDAC immunohistochemically, and observed weak expression of SOX2 in pancreatic intraepithelial neoplasia (PanIN-3) lesions. They also observed relatively high and frequent expression in invasive and poorly differentiated PDAC. Later, it was shown that at the mRNA level, SOX2 expression driven by hedgehog-EGFR signaling is necessary for tumor-initiating pancreatic cancer cells[55]. Very recently, the molecular mechanism underlying SOX2 regulation of pancreatic cancer stemness has been elucidated. Using primary human cancer tissues and cell lines (L3.6, Bxpc3, CFPAC-1, Panc1 and Panc04.03), our group demonstrated a critical role for SOX2 in promoting cell proliferation, dedifferentiation and impartment of stem cell-like features to pancreatic cancer cells[38]. In particular, SOX2 gene suppression arrested cells at the G_1 phase and its overexpression alone was sufficient to drive cell proliferation by facilitating G_1/S transition. Mechanistically, G_1 arrest in SOX2 knockdown cells is associated with a marked induction of $p21^{Cip1}$ and $p27^{Kip1}$, two key cyclin/CDK inhibitors, whereas SOX2 overexpression induces G_1/S-specific cyclin D3 expression. All of three cell cycle regulators were identified as *bona fide* SOX2 regulatory targets. SOX2 also confers pancreatic cancer cell stemness and its overexpression alone is sufficient to drive sphere-formation and expression of CSC markers[7,38,45,56], as well as induce EMT drivers such as Snail, Slug and Twist (Figure 2). Consistently, loss of *miR-145* elevates SOX2 and impairs differentiation in pancreatic tumors[57].

It is now evident that the core stem cell factors OCT4[16], SOX2[58], and NANOG[59] play essential roles in the maintenance of pluripotency and self-renewal of ESCs, ASCs, iPSCs and CSCs. These stem cell factors promote self-renewal by interacting with other transcription factors (Stat3, Hesx1, Zic3), critical cell signaling molecules (Hedgehog, TCF3, FGF2, LEFTY2)[60], and have been found aberrantly expressed in several types of human tumors including pancreatic cancer[61-63]. Although ESCs and CSCs share the property of self-renewal, they also reveal distinct features in that ESCs favor differentiation, whereas CSCs are more biased toward proliferation and inhibition of apoptosis. In particular, SOX2 has demonstrated OCT4 and/or NANOG independent activity in pancreatic cancer cells in promoting cell pro-liferation, survival, and/or de-differentiation[38]. Recent work by Polvani *et al*[47] further supports this statement demonstrating that OCT4 silencing reduces OCT4 and increases NANOG, but does not alter SOX2 expression.

CSCs AS TARGET FOR CANCER THERAPY

SOX2 immunoreactivity has been demonstrated in PanIN lesions, as well as moderately and poorly differentiated tumors, which is consistent with previous reports showing an enrichment of SOX2 in pancreatic CSCs[64], as well as a decreased expression after anti-ESCs therapies[55,65]. Since SOX2 appears to be a key factor aberrantly expressed in PDAC and confers CSCs-like properties[38], targeting SOX2 or its upstream regulator(s) may be exploited for therapeutic purposes. Recent reports demonstrate that using poly (lactide-co-glucolide) to knockdown DCLK1 results in an increase in miR-145 associated with decreased puripotency factors including SOX2, and consequently, tumor growth arrest in xenografts[57,66]. Lastly, data from Sobrevals *et al*[67] elucidates the relevance of uPAR-controlled oncolytic adenoviruses in the elimination of pancreatic CSCs. Along these lines, C-Met inhibitors have been demonstrated to overcome gemcitabine resistance and stem cell signaling through downregulation of CSC markers including SOX2[65]. Strategies to target CSCs for cancer therapy have been proposed and are under investigation. For instance, metformin directed against pancreatic CSC has been shown to reduce tumor burden and prevent disease progression[68]. Disulfiram, an ALDH inhibitor, was tested *in vitro* and *in vivo* demonstrated a capacity to suppress pancreatic CSCs[69]. Promising results suggest that HAb18G/CD47 or Phospho-valproic acid (MDC-1112) could also be a promising target in pancreatic cancer surrogating anti-STAT3 therapies[70,71]. More recently, HAb18G/CD147 has been identified as another promising therapeutic target for highly aggressive pancreatic cancer and a surrogate marker in the STAT3-targeted molecular therapies, such as by phospho-valproic acid (MDC-1112), a novel valproic acid derivative. Since targeting CSCs has been demonstrated to be a viable therapeutic strategy against pancreatic cancer, a better undertanding of OCT4, NANOG and particularly SOX2 on their expression and regulatory circuitry in PDAC will facilitate the design of individualized therapies for PDAC patients.

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Management of borderline and locally advanced pancreatic cancer: Where do we stand?

Jin He, Andrew J Page, Matthew Weiss, Christopher L Wolfgang, Joseph M Herman, Timothy M Pawlik

Jin He, Andrew J Page, Matthew Weiss, Christopher L Wolfgang, Timothy M Pawlik, Department of Surgery, Johns Hopkins Hospital, Baltimore, MD 21287, United States
Joseph M Herman, Department of Radiation Oncology, Johns Hopkins Hospital, Baltimore, MD 21287, United States
Author contributions: All authors contributed equally to the production of this manuscript.
Correspondence to: Timothy M Pawlik, MD, MPH, PhD, Professor, Department of Surgery, Johns Hopkins Hospital, 600 N. Wolfe Street, Blalock 688, Baltimore, MD 21287, United States. tpawlik1@jhmi.edu
Telephone: +1-410-5022387 Fax: +1-410-5022388
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Abstract

Many patients with pancreas cancer present with locally advanced pancreatic cancer (LAPC). The principle tools used for diagnosis and staging of LAPC include endoscopic ultrasound, axial imaging with computed tomography and magnetic resonance imaging, and diagnostic laparoscopy. The definition of resectability has historically been vague, as there is considerable debate and controversy as to the definition of LAPC. For the patient with LAPC, there is some level of involvement of the surrounding vascular structures, which include the superior mesenteric artery, celiac axis, hepatic artery, superior mesenteric vein, or portal vein. When feasible, most surgeons would recommend possible surgical resection for patients with borderline LAPC, with the goal of an R0 resection. For initially unresectable LAPC, neoadjuvant should be strongly considered. Specifically, these patients should be offered neoadjuvant therapy, and the tumor should be assessed for possible response and eventual resection. The efficacy of neoadjuvant therapy with this approach as a bridge to potential curative resection is broad, ranging from 3%-79%. The different modalities of neoadjuvant therapy include single or multi-agent chemotherapy combined with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation. This review focuses on patients with LAPC and addresses recent advances and controversies in the field.

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Key words: Pancreas; Locally advanced; Chemotherapy; Radiation; Irreversible electroporation; Pancreatic cancer

Core tip: While the management of resectable patients is surgery (with or without neoadjuvant therapy), and the management of grossly metastatic patients is palliative with systemic chemotherapy with or without radiation, there is an intermediate subset of patients with locally advanced disease which is less straightforward. This review focuses on this unique population of patients with locally advanced pancreatic adenocarcinoma and addresses recent advances and controversies in this field.

INTRODUCTION

Pancreatic adenocarcinoma is a lethal disease with a high metastatic potential. In 2012, there were an estimated 43920 patients diagnosed with pancreas cancer, and 37390 were expected to die from their disease[1]. The only available potential cure for pancreas cancer is surgical resection, with only 15%-20% of patients presenting with

pancreas cancer being candidates for resection. For those patients that go onto resection, the 5-year survival ranges from 15%-20%, whereas the 5-year survival for all pancreas cancer patients combined is 3%[1,2].

The factors that lead to the overall dismal prognosis of pancreatic cancer are multiple and varied, making management a challenge. These factors include absence of nonspecific symptoms that leads to delayed diagnosis, biological aggressiveness which is resistant to chemotherapy, and surgical considerations which can be technically demanding[3,4]. While the management of resectable patients is surgery (with or without neoadjuvant therapy), and the management of grossly metastatic patients is palliative with systemic chemotherapy with or without radiation, there is an intermediate subset of patients with locally advanced disease which is less straightforward. This review focuses on this unique population of patients with locally advanced pancreatic adenocarcinoma and addresses recent advances and controversies in this field.

DIAGNOSIS OF LOCALLY ADVANCED PANCREAS CANCER

As technology has evolved, the tools available to evaluate locally advanced pancreas cancer (LAPC) have become more accurate. The principle tools used for diagnosis and staging of LAPC include endoscopic ultrasound (EUS), axial imaging with computed tomography (CT) and magnetic resonance imaging (MRI), and diagnostic laparoscopy[5]. Endoscopic ultrasound provides images of the pancreas and surrounding vessels, and in particular allows for tissue diagnosis with the capability to biopsy. Endoscopic retrograde cholangiopancreatography (ERCP) can be performed at the same time if there is an indication to stent the common bile duct. Therefore, EUS can diagnose the tumor with biopsy, stage the tumor by size and vascular involvement, and use ERCP to therapeutically stent the common bile duct, should it be necessary.

CT with intravenous contrast provides multiplanar, high-resolution, three-dimensional images of the pancreatic tumor, its surrounding vascular structures, and possible lymphadenopathy and liver metastases. Warshaw *et al*[6] demonstrated that more than 90% of patients deemed unresectable by CT are actually unresectable at operation. MRI can also be used to assess extent of tumor involvement and has shown to be equivalent to CT[7]. Difficulties with CT and MRI include measuring response to treatment, particularly in patients who have undergone treatment with radiation therapy[8]. However, with developments in imaging technology, assessment of staging and tumor response is likely to only improve for the patient with pancreatic cancer.

Another pitfall for current axial imaging is the limitation to incompletely visualize potentially small (1-2 mm) tumor deposits[9]. This is critical to the management of pancreas cancer, as patients with extra-pancreatic disease have the same dismal prognosis as those with metastatic disease, and these patients should not be put at risk from a potentially morbid laparotomy or pancreatectomy. This problem can be addressed using diagnostic laparoscopy to directly visualize the intra-abdominal contents, in particular the liver and peritoneum. Patients who should be considered for diagnostic laparoscopy prior to laparotomy are those patients with possible undetectable metastatic disease, *i.e.*, primary tumors > 3 cm, marked weight loss, equivocal radiological findings, and elevated levels of carbohydrate antigen 19-9 (CA19-9)[10].

Definition and ambiguity of LAPC

The biology of LAPC is unique in that the tumor is confined locoregionally, without evidence of distant macrometastatic disease. The precise molecular mechanisms responsible for this behavior are unclear, but involve a preservation of the epithelial cell type *vs* de-differentiating into the mesenchymal phenotype responsible for distant spread[11]. Specific signals involved in this cell-type transformation include transforming growth factor beta (TGFβ), E-cadherin, N-cadherin, K-ras, and Snail, along with the chemokine CXCL12[12-14]. On a macroscopic level, LAPC has an anatomic definition and is represented by two subclasses of aggressive pancreas cancer - borderline resectable LAPC and unresectable LAPC. For the patient with LAPC, there is some level of involvement of the surrounding vascular structures, which include the superior mesenteric artery (SMA), celiac axis, hepatic artery, superior mesenteric vein (SMV), or portal vein (PV). Depending on the extent of vessel involvement, and whether the associated vascular structures are amenable to reconstruction in conjunction with resection of the tumor, defines whether the LAPC is deemed borderline resectable or unresectable (Figures 1 and 2).

Unfortunately, this definition of resectability has historically been vague, as there is considerable debate and controversy as to which patients are truly deemed resectable. Factors that contribute to this confusion are multiple, and include subjective interpretation of cross-sectional imaging, technical/surgical ability, and overall institutional experience. Because of the lack of consensus of a true definition of LAPC, the literature available for LAPC is not standardized, and generalizations and conclusions about the management of LAPC have suffered[5,8].

To address the lack of general consensus on a definition of LAPC, three guideline statements have recently been proposed. These include guideline proposals by the National Comprehensive Cancer Network (NCCN), The University of Texas M.D. Anderson Cancer Center (MDACC), and Americas Hepato-Pancreato-Biliary Association (AHPBA). All three guidelines include the aforementioned tumor relationships to vascular structures, however there is variability in the definition of the tumor-vascular involvement. Further, some guidelines have added additional subset criteria to more specifically define the population of patients with LAPC. The MDACC guidelines were supplemented with three sub-

Figure 1 Computed tomography of locally advanced pancreatic cancer. Encasement is defined as greater than 180-degree involvement of the major vessels. A: Celiac axis is encased by locally advanced pancreatic cancer (arrow); B: Superior mesenteric artery and the replaced right hepatic artery are encased by pancreatic cancer (arrow); C: The portal vein and its confluence with splenic vein are encased by pancreatic cancer (arrow).
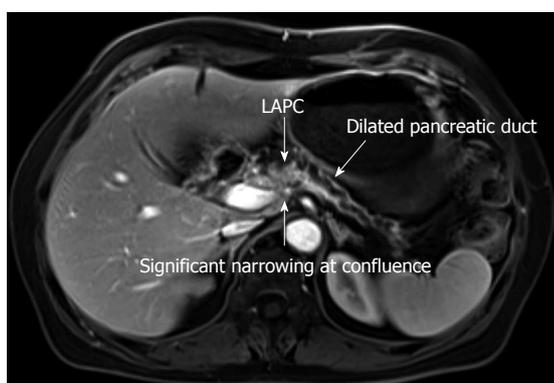
Figure 2 Magnetic resonance imaging of locally advanced pancreas cancer with vascular invasion and dilated pancreatic duct. LAPC: Locally advanced pancreatic cancer.

classifications of borderline resectable-types A, B and C. MDACC type A patients are only those patients with local, tumor-artery abutment. Type B patients are those with questionable extrapancreatic metastatic disease. Further defined, these type B patients are considered "oncologically borderline resectable" secondary to prior exploration which the original tumor was considered unresectable, a prior biopsy confirmed regional lymph node metastasis, or there is imaging concerning for liver metastases or high CA19-9. Type C patients are those defined as having a marginal pretreatment performance status[15].

The Alliance for Clinical Trials in Oncology (Alliance) recently initiated a multi-institutional trial to examine the use of neoadjuvant for LAPC in a single arm pilot study[16]. This study also seeks to address the lack of standardization in the definition of LAPC and to establish a research infrastructure that will create consensus around what constitutes borderline and unresectable LAPC. In the Alliance proposal, the definition of a borderline resectable pancreas cancer has an objective description of the tumor-vascular relationships, while omitting more subjective terms like abutment and encasement. These guidelines should create uniformity in how investigators define LAPC both for protocol and non-protocol based therapies[16] (Table 1).

A multi-disciplinary approach is highly recommended

in the treatment of patients with LAPC, and can assist with arriving at a consensus recommendation for the treatment of patients with advanced disease. By bringing together medical oncologists, surgeons, radiologists, radiation oncologists, and other patient advocates, treatment plans for the patient with LAPC can be discussed and planned[17]. The complexity of LAPC is best managed by this multidisciplinary team of physicians working in concert to deliver individualized care for each patient[18]. The importance of a multi-modal, inter-disciplinary approach has been demonstrated in our own multidisciplinary pancreatic cancer clinic at Johns Hopkins, where we noted that 25% of patients seen in this setting had a significant change in their diagnosis or treatment[18].

BORDERLINE LAPC

Surgical resection of LAPC

Resection of the surrounding vascular structures for LAPC has been described since the 1970s. Fortner et al[19] described these "regional pancreatectomies" as type 1 (venous resection) and type 2 (arterial resection). These early reports demonstrated significant morbidity and mortality, and given the potential for likely systemic disease, combined tumor and vascular resection fell out of favor[20]. Despite early hesitation with combined resection of tumor and surrounding vascular structures, there is now growing enthusiasm for these more aggressive surgeries. One of the most controversial topics for these patients is the role of margin status after resection. This is particularly relevant for the patient with borderline LAPC, as vascular involvement of surrounding structures, even when technically achievable, may predispose to a positive resection margin.

Multiple reports suggest that margin status after resection of pancreas cancer influences survival[21,22]. However, other data demonstrate that margin status does not correlate with survival[23,24]. There are a variety of factors that have led to this ambiguity. One of the strongest influences fueling this discrepancy has been the lack of standardization of pathologic technique, i.e., truly defining a "positive microscopic margin."[25]. This is evident from multiple large studies which demonstrate the rate of R1 involvement for pancreas cancer varies between

Table 1 Difference of definitions of anatomic borderline resectable pancreatic cancers from different sources

Tumor-vessel relationship on computed tomography	NCCN	MDACC	AHPBA/SSO/SSAT	Alliance
Superior mesenteric vein/ portal vein	Severely narrowed or occluded with possibility of reconstruction	Occluded with possibility of reconstruction	Abutment or encasement or occlusion with possibility of reconstruction	Interface between tumor and vessel > 180°, and or reconstructable
SMA	Abutment	Abutment	Abutment	Interface between tumor and vessel < 180°
Celiac axis	No abutment or encasement	Abutment	No abutment or encasement	Reconstructable interface
Common hepatic artery	Abutment or short segment encasement	Abutment or short segment encasement	Abutment or short segment encasement	Interface between tumor and vessel < 180°

Abutment, ≤ 180° or ≤ 50% of the vessel circumference; encasement, ≥ 180° or ≥ 50% of the vessel circumference. MDACC: Anderson Cancer Center; NCCN: National Comprehensive Cancer Network; AHPBA: Hepato-Pancreato-Biliary Association; SMA: Superior mesenteric artery.

20% and 80%, despite other clinicopathological variables being similar[26,27]. Fortunately, there have been improvements in standardization, and consensus is growing in the pathology community regarding how to examine the pathology specimen[28].

Other groups have also examined the effect of margin status from the surgical perspectives. Butturini *et al*[29] pooled hazard ratios of the effects of adjuvant therapy for resected patients, and compared the disease specific survival with their margin status. As part of their subset analysis, the authors concluded that resection margin (R0 *vs* R1) involvement was not a statistically significant prognostic factor, with a median survival of 14.1 mo for patients with an R1 resection compared with 15.9 mo for patients with R0 resections (*P* = 0.24).

From a technical standpoint, superior mesenteric vein and portal vein involvement by LAPC can be performed safely if resected and reconstructed at high-volume centers[30]. Reconstruction of the SMV/PV can be performed in a variety of ways depending on the degree of involvement. Patch or primary closure can be done for partial involvement, with patch reconstruction often done using the greater saphenous vein. Segmental reconstruction of the SMV can be performed with an interposition vein graft using the internal jugular, renal vein or superficial femoral vein[31,32]. Raut *et al*[24] examined 360 patients after pancreatectomy, of which 130 underwent SMV/PV reconstruction. Those patients who underwent vascular reconstruction had more R1 than R0 resections compared with those that did not have vascular reconstruction (HR = 2.00, *P* = 0.015). However, on multivariate analysis, there was no difference in survival between the R1 and R0 groups, leading the authors to conclude that not only was there no difference in patient survival based on R status, but venous reconstruction also did not predispose to worse disease-specific survival.

Compared with venous reconstruction, arterial involvement is probably more technically demanding. If an interposition graft is required, this can be done with polytetrafluoroethylene (PTFE) graft or saphenous vein[33]. Bockhorn *et al*[34] has reported one of the largest series to examine pancreatic resection with simultaneous arterial resection and reconstruction (*n* = 29); these au-

thors found no difference in overall disease specific survival for patients who underwent arterial reconstruction *vs* those patients that had pancreatectomy alone (14.0 mo *vs* 15.8 mo respectively, *P* = 0.152). Both resection groups independently had better survival than the non-resected patients who only underwent palliative bypass (7.5 mo, *P* < 0.05 for both groups)[34].

Therefore, if feasible, most surgeons would recommend possible surgical resection for patients with borderline LAPC, with the goal of an R0 resection for all cases. While vascular resection with reconstruction is safe, patient selection is paramount. Those patients who cannot tolerate combined pancreatectomy and vascular reconstruction would benefit more from palliative bypass or no surgery at all.

BORDERLINE LAPC AND NEOADJUVANT THERAPY

Because of the dismal prognosis of pancreatic cancer, in particular those with borderline LAPC which may have a more aggressive biology, there is a growing body of literature to suggest that there is a potential role for neoadjuvant therapy to treat micrometastatic disease with chemotherapy, as well as treat local disease with radiation[35,36]. The rationale for neoadjuvant therapy for patients with borderline and LAPC is multifold. First, the chance of delivering full-dose chemotherapy with or without radiation is much better if given prior to surgery because of the potential delay in getting to treatment after a complex pancreatic resection. Second, neoadjuvant therapies provide insight into the biology of the disease, and can spare patients who progress or develop distant metastasis during treatment from undergoing a major surgery that would not be curative. Next, neoadjuvant therapies have the potential to downstage borderline resectable disease to the point of not requiring vascular reconstruction and/or increasing R0 resection. Lastly, preoperative therapy could be more effective than post resection therapy because the resected tumor bed may have decreased oxygenation and decreased drug delivery[37]. While there are benefits of neoadjuvant therapy for borderline LAPC,

these benefits must be weighed against the risks, which include delaying time to potentially curative surgery and significant time and side-effects for patients with limited life expectancies.

There are only retrospective studies with subsets of borderline LAPC, and a few smaller prospective studies examining the role of neoadjuvant therapies for borderline LAPC[15,38-40]. Patel *et al*[41] prospectively examined 17 patients with borderline LAPC for patients that were treated with combined chemoradiation, with 64% proceeding to surgery with 89% achieving an R0 resection. Stokes *et al*[40] also prospectively examined 40 borderline LAPC, also with combined chemoradiation, with 40% of patients proceeding to surgery, with 88% with an R0 resection, and median survival at 23 mo.

INITIALLY UNRESECTABLE LAPC AND NEOADJUVANT THERAPY

For initially unresectable LAPC, *i.e.*, those tumors with significant vascular involvement that involves a significant portion of the SMV or SMA, neoadjuvant therapy should be offered, and the tumor should be assessed for possible response and eventual resection. The efficacy of neoadjuvant therapy with this approach as a bridge to potential curative resection is broad, ranging from 3%-79%[42-44]. The different modalities of neoadjuvant therapy include single or multi-agent chemotherapy combined with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation.

Combined chemotherapy with radiation

5-flourouracil (5-FU) infusion with radiation therapy has shown utility in many gastrointestinal cancers, and is used in the management of unresectable LAPC. One of the first studies to demonstrate the synergistic effects of 5-FU with radiation was the Gastrointestinal Study Group (GITSG) trial in 1981 that prospectively examined unresectable LAPC patients, randomly assigning 106 patients to three different treatments: radiation (60 Gy) alone, *vs* concurrent radiation (40 Gy) plus bolus 5-FU, *vs* higher dose concurrent radiation (60 Gy) plus bolus 5-FU[45]. The radiation alone group demonstrated poor 1-year survival (11%) *vs* 36% in the higher dose concurrent radiation group, and 38% in the concurrent lower radiation group. Other trials have demonstrated this synergistic and radiosensitizing effect of combined 5-FU with radiation[46-48]. Contrary to successes of these groups and the GITSG trials using combined 5-FU with radiation, a trial from the Eastern Cooperative Oncology Group (ECOG) randomized 91 patients with unresectable LAPC to either radiation (40 Gy) plus concurrent bolus 5-FU, followed by weekly maintenance 5-FU, *vs* 5-FU alone, and found no differences in survival (8.2 mo *vs* 8.3 mo)[49,50]. Despite the conflicting success of combined 5-FU/radiation therapy, this radiosensitization treatment modality has become an established approach to management of the patient with LAPC[51].

In an effort to capitalize on the benefits of combined 5-FU and radiation therapies, yet avoid the toxic side effects of 5-FU therapy, the oral formulation of 5-FU, capecitabine, has been introduced into many trials. To date there are multiple studies, albeit only a few prospective trials, that demonstrate that capecitabine can effectively replace infusional 5-FU in the setting of LAPC[52-54].

As the potential utility of combined 5-FU/radiation therapies was being recognized for LAPC, gemcitabine based regimens were gaining acceptance in the management of metastatic pancreas cancer[55]. Therefore, gemcitabine combined with radiation gained interest as a potential agent to study in the management of LAPC. Unfortunately, early phase I trials using gemcitabine with radiation were fraught with toxicities unlike the 5-FU based therapies, and required improvements in delivery of radiation[56-58]. As the toxicities of combined gemcitabine and radiation therapy became more manageable, studies were designed to compare the established 5-FU and radiation therapy with gemcitabine combined with radiation for LAPC.

Three large prospective studies were designed with this hypothesis in mind. The Federation Francophone de Cancerologie Digestive and Societie Francaise de Radiotherapie Oncologique (FFCD-SFRO) trial published in 2008 showed improved survival for those patients treated with gemcitabine alone *vs* combined radiotherapy with 5-FU (13.0 mo *vs* 8.6 mo, P = 0.03)[59]. The ECOG E4201 study, published 3 years after the FFCD-SFRO study, compared gemcitabine plus radiation with gemcitabine alone, and found improved survival in the combined group (11.1 mo *vs* 9.2 mo, P = 0.017), although there was more toxic side effects in the combined group[60]. The Taipei trial, which compared combined gemcitabine and radiation with combined 5-FU and radiation, concluded that combined gemcitabine and radiation therapy had improved overall survival (14.5 mo *vs* 6.7 mo, P = 0.027)[48]. These large series solidified the utility of gemcitabine based chemoradiation as an acceptable option for patients with LAPC.

A recent trial has further examined 5-FU combined therapies using capecitabine, and compared efficacy with gemcitabine-based chemoradiotherapy. Mukherjee *et al*[61] in the Selective Chemoradiation in Advanced Localized Pancreatic Cancer (SCALOP) study, examined 74 patients with LAPC who were randomly assigned gemcitabine or capecitabine. These authors found that the capecitabine treated patients had improved survival over the gemcitabine treated patients (15.2 mo *vs* 13.4 mo, P = 0.012). Furthermore, the gemcitabine treated patients had more toxic non-hematologic (10 *vs* 4, P = 0.12) and hematologic side effects (7 *vs* 0, P = 0.008).

Just as the combined chemotherapy and radiation algorithm has focused on changing the chemotherapeutic agent in an attempt to maximize survival benefit and minimize toxicity, other studies have examined the different radiation delivery modalities. The earlier combined chemoradiation treatments incorporated external beam
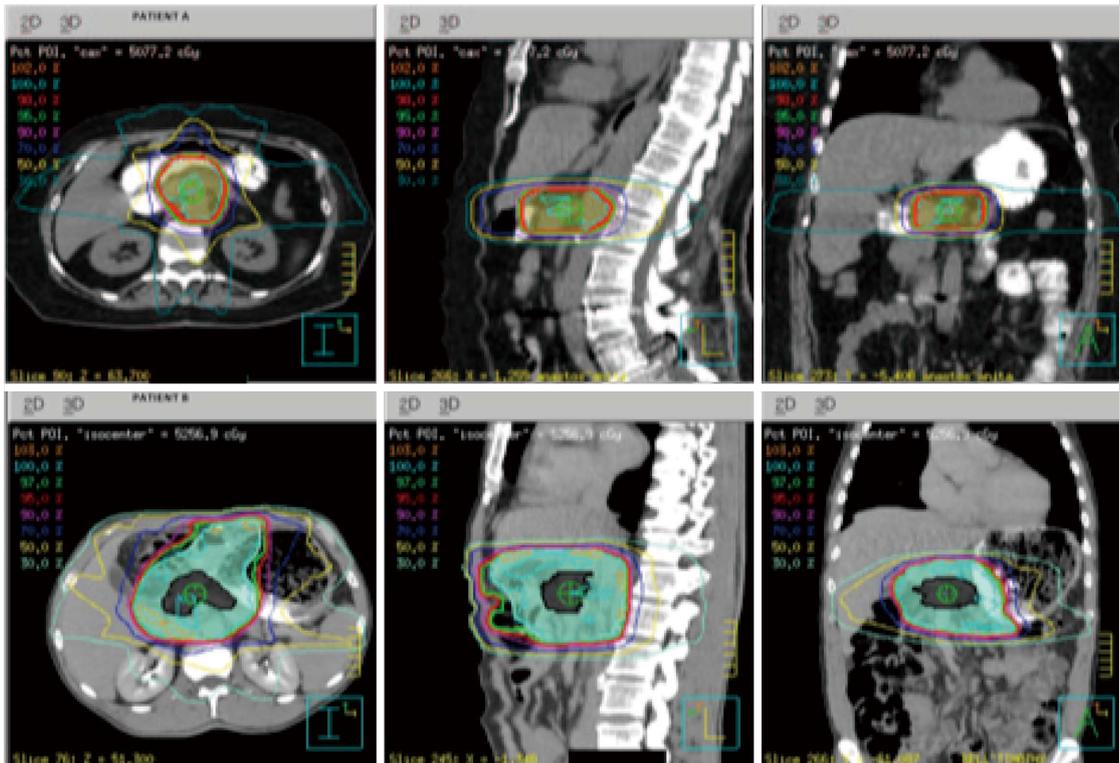
Figure 3 Depiction of stereotactic body radiation plan using computed tomography. Typically the tumor is expanded 2-3 mm to account for set up error microscopic extension and set-up error planning treatment volume. In the lower panel, (patient b) this represents a plan integrating intensity modulated radiation therapy (IMRT) where the tumor is expanded 1-3 cm to cover the tumor and peripancreatic lymph nodes. Stereotactic body radiation is often delivered over 1-5 d without chemotherapy. IMRT is delivered over 5-6 wk with concurrent chemotherapy.

radiation (EBRT). Since the 1980s, other delivery systems have developed with the integration of 3-D conformal radiation and subsequently intensity modulated radiation therapy (IMRT) and stereotactic body radiation (SBRT). Conventional EBRT has limitations in the amount of radiation that can be delivered to the pancreas tumor secondary to damage to the surrounding GI tract and other healthy tissues. In addition, EBRT also usually requires a large number of treatments given over 5-6 wk. SBRT and IMRT can deliver more focused radiation therapy to the tumor plus a margin, and thus limit dose to normal bowel resulting in less toxicity and dose escalation to the tumor. IMRT represents a further advancement from conformal EBRT. By utilizing 3-D conformations of a tumor target, radiation *via* IMRT can be delivered in smaller divisions of beams (beamlets), while both sparing healthy tissue and having the capacity to up or down regulate the intensity of the target directed beamlets[62]. SBRT enables delivery of even more precise and large doses of radiation to the pancreas tumor plus a small margin (usually 2-3 mm) because of the rapid dose fall-off beyond the treated volumes. SBRT is also usually given in 1-5 fractions, far fewer than EBRT (10-30)[63] (Figure 3).

Because of the toxicities which may arise during chemoradiation, combined with the overall poor survival of LAPC, it is critical in the multidisciplinary management of LAPC to identify which patients may experience worse outcomes. Rudra *et al*[64] identified pretreatment performance status and CA19-9 levels, along with treatment interruption as prognostic factors for patients with LAPC treated with chemoradiation. These authors proposed that patients should be identified with these poor outcome features prior to treatment, and consider other therapies such as chemotherapy alone or supportive care for patients with poor performance status.

Chemotherapy alone

Chemotherapy alone represents another management strategy for unresectable LAPC. The primary chemotherapy only regimens include gemcitabine alone; gemcitabine doublet therapy with oxaliplatin, cisplatin, erltoinib, or capecitabine; or triplet therapy with oxaliplatin and erlotinib, or oxalplatin and bevacizumab. Other non-gemcitabine-based regimens include irinotecan with docetaxel[65].

Multiple trials have examined patients with LAPC, comparing gemcitabine alone with various gemcitabine doublet therapies. Louvet *et al*[66], in the GERCORD and GISCAD trials found no difference in overall survival (9.0 mo *vs* 7.1 mo, *P* = 0.13) using gemcitabine alone *vs* doublet therapies. Similar survival was also seen when gemcitabine was compared with and without tipifanib (193 d *vs* 182 d, *P* = 0.75)[67]. Other groups have examined gemcitabine combined with irinotecan (IRINOGEM), and while time-to-progression initially showed promise for the IRINOGEM treated group *vs* gemcitabine alone

Table 2 Summary of recent chemotherapy trials for locally advanced pancreatic cancer

CHEMO trials	Component	Median survival	*P* value
GERCORD/GISCAD[66]	Gem ± oxaliplatin	9.0 mo *vs* 7.1 mo	0.13
Van Cutsem *et al*[67]	Gem ± tipifarnib	193 d *vs* 182 d	0.75
IRINOGEM[68]	Gem ± irinotecan	6.3 mo *vs* 6.6 mo	0.79
Von Hoff *et al*[69]	Gem ± *nab*-paclitaxel	8.5 mo *vs* 6.7 mo	< 0.001
PRODIGE[70]	Gem *vs* FOLFIRINOX	6.8 mo *vs* 11.1 mo	< 0.001

CHEMO: Chemotherapy; Gem: Gemcitabine.

(median 7.7 mo *vs* 3.9 mo, *P* value not reported), there was no difference in overall survival (6.3 mo *vs* 6.6 mo, *P* = 0.789)[68]. Von Hoff *et al*[69] using combined gemcitabine with nab-paclitaxel *vs* gemcitabine monotherapy demonstrated a survival benefit in patients with metastatic pancreas cancer (8.5 mo *vs* 6.7 mo, *P* < 0.001). The application of this regimen for LAPC is not known. In summary for gemcitabine-based chemotherapies, in the setting of LAPC, there are no prospective data to suggest that gemcitabine doublet, or even triplet therapy improves overall survival over monochemotherapy using gemcitabine alone.

While multiple agent gemcitabine based chemotherapies have not shown direct promise in the management of LAPC, other non-gemcitabine based regimens are being explored. The multiple agent therapy of 5-FU/leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) has recently shown promise in the management of metastatic pancreas cancer in the PRODIGE trial, and is being studied in the context of LAPC[70]. In three retrospective reviews of FOLFIRNOX for LAPC, partial response rates ranged from 25%-40%[71-73]. Other multiple agent therapies like oxaliplatin, 5-FU, and folinic acid (FOLF-OX-6), and agents like 5-FU plus leucovorin plus irinotecan (FOLFIRI), are also being studied as potential agents to improve outcomes in unresectable LAPC[74,75]. While some progress has been shown using chemotherapy alone regimens for LAPC, the specific treatment with best results has yet to be determined (Table 2).

Chemotherapy followed by chemoradiotherapy
An additional treatment algorithm for LAPC is the use of chemotherapy followed by chemoradiotherapy. The specific goal of this treatment is to select the patients treated with chemotherapy who will benefit from chemoradiotherapy, and also to select those who have not progressed following the initiation of chemotherapy. The earliest and one of the largest studies to examine this mode of therapy was the Groupe Cooperatuer Multidsisciplinaire en Oncologie (GERCOR). This group retrospectively reviewed 181 patients with LAPC who had been treated with gemcitabine-based chemotherapy followed by chemoradiotherapy using 5-FU in continuous infusion[76]. Fifty-three patients developed metastases in the first 3 mo of chemotherapy and were subsequently not eligible for chemoradiation. In the remaining 128 patients who did not progress, 56 continued with chemotherapy alone with overall survival of 11.7 mo. The other 72 patients

received chemoradiation, with overall survival of 15.0 mo (*P* < 0.01).

Another retrospective study by the University of Texas M.D. Anderson Cancer Center examined consecutive patients with LAPC who had received treatment with chemoradiation or induction chemotherapy followed by chemoradiotherapy[77]. Of the 323 patients in this study, 76 received a median of 2.5 mo of gemcitabine prior to chemoradiation. Those who underwent chemotherapy prior to combined chemoradiation had improved median overall survival (11.9 mo *vs* 8.5 mo, *P* < 0.001), and also demonstrated improved progression free survival (6.4 mo *vs* 4.2 mo, *P* < 0.001).

While the use of chemotherapy followed by chemoradiation has shown early promise in the management of LAPC, phase Ⅱ/Ⅲ studies are needed. The ECOG 1200 phase Ⅱ trial was initially designed to evaluate the safety of borderline resectable LAPC using the algorithm of chemotherapy followed by chemoradiation, but was closed early because of low recruitment[44].

In summary of the treatments modalities available for unresectable LAPC, a recent retrospective review by Lloyd *et al*[65] compared outcomes based on combined chemotherapy with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation. While the sample size was small (*n* = 115), and included borderline and unresectable LAPC, the authors concluded on multivariate analysis that chemotherapy followed by chemotherapy with radiation was associated with improved overall survival over chemotherapy alone or combined chemotherapy with radiation (median survival 21.5 mo *vs* 13.9 mo and 12.5 mo respectively, *P* < 0.05).

Locoregional therapy with irreversible electroporation
For some patients with LAPC, irreversible electroporation (IRE) has shown promise in downstaging and prolonging survival. IRE is a non-thermal modality that uses high voltage and low energy direct current to increase cell membrane permeability and effectively create defects in cell membranes, resulting in loss of homeostasis and subsequent cell death. IRE has minimal effect on blood vessel scaffolding, which is crucial and particularly relevant for LAPC, as surrounding vascular involvement may be present[78,79].

The NanoKnife®IRE system has been commercially available since 2009 and is FDA-approved to treat soft tissue tumors. The safety of IRE use in the pancreas has been shown in swine models with rapid resolution
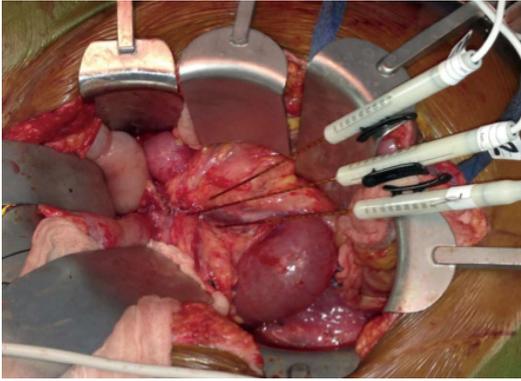
Figure 4 An intraoperative image of *in situ* irreversible electroporation being used in a patient with locally advanced pancreatic cancer. Three probes are placed around the tumor which is encasing the superior mesenteric vein causing complete occlusion plus superior mesenteric artery involvement.

Figure 5 This is the representative base unit and generator for irreversible electroporation, manufactured by AngioDynamics, Latham, NY.

of pancreatitis and preservation of vascular structures. Ablation effects can be achieved at a median size of 3 cm with 3000 volts setting of the NanoKnife®IRE system[78]. Usually, 2-4 probes of the NanoKnife®IRE system are used to treat LAPC. The probes are placed using intraoperative ultrasound guidance. In a retrospective series of patients treated at a single institution, Martin *et al*[80]. applied this new device and demonstrated in unresectable LAPC that IRE can improve both local (14 mo *vs* 6 mo, *P* = 0.001) and distant progression free survival (15 mo *vs* 9 mo, *P* = 0.02), compared with systemic therapy and chemoradiation. Overall survival for patients treated with IRE was also improved compared with patients treated with chemotherapy alone or chemoradiation (20 mo *vs* 13 mo, *P* = 0.03, exact chemoradiation regimens not specified) (Figures 4 and 5).

IRE can be administered percutaneously under imaging guidance, thereby avoiding the morbidity of a laparotomy. Narayanan *et al*[81] reported the results of 11 patients treated with IRE for LAPC. In this study, prior to IRE, all patients had received some form of chemoradiation, though the exact regimen was not specified. Patients were selected for IRE if they were not candidates for, or were intolerant of chemotherapy or radiation. The procedure was performed under general anesthesia, with CT guidance, and electrodes were placed at a maximum of 2.2 cm apart. Post treatment, all patients demonstrated patent vasculature in the treatment zone and there were no deaths related to the procedure. Two patients underwent partial responses leading to eventual resection 4 and 5 mo post IRE, with one of these patients demonstrating a complete response. Both patients remained disease free at 11 and 14 mo. At our institution, we often maximize both systemic and local therapy (radiation), then in well selected patients, we attempt surgical resection with IRE in an attempt to sterilize surgical margins or treat the tumor intra-operatively if found to be unresectable.

CONCLUSION

LAPC is a biologically aggressive cancer with unique characteristics, prognosis, and management strategies that differentiate this pancreatic tumor from resectable cancer and metastatic disease. The only means to potentially cure LAPC is by maximizing upfront systemic and local therapy followed by a margin negative surgical resection. At Johns Hopkins Hospital, we recommend tailoring therapy to maximize the chance to offer the patient a chance at surgical resection. In general, if LAPC is preoperatively identified as not resectable, then we proceed down a pathway of local control with radiation therapy combined with systemic control with chemotherapy. After chemoradiation, we restage and re-evaluate for possible resection, with IRE as an alternative therapy for the unresectable LAPC.

Unfortunately, surgical and chemoradiation protocols have suffered from lack of consensus on what truly defines both a resectable LAPC and a positive resection margin. But with growing adoption of consensus guidelines, and the incorporation of improved systemic therapies and local therapeutic options with decreased side effects, progress is being made in identifying which patients with LAPC can truly benefit from surgical resection.

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2014 ADVANCES IN PANCREATIC CANCER

Systematic review of novel ablative methods in locally advanced pancreatic cancer

Margaret G Keane, Konstantinos Bramis, Stephen P Pereira, Giuseppe K Fusai

Margaret G Keane, Stephen P Pereira, Institute for Liver and Digestive Health, University College London, Royal Free Campus, London NW3 2PF, United Kingdom

Konstantinos Bramis, Giuseppe K Fusai, HPB and Liver Transplant Unit, Royal Free Hospital, London NW3 2QG, United Kingdom

Correspondence to: Giuseppe K Fusai, MS, FRCS, HPB and Liver Transplant Unit, Royal Free Hospital, London NW3 2QG, United Kingdom. g.fusai@nhs.net
Telephone: +44-20-77940500 Fax: +44-20-78302960

Abstract

Unresectable locally advanced pancreatic cancer with or without metastatic disease is associated with a very poor prognosis. Current standard therapy is limited to chemotherapy or chemoradiotherapy. Few regimens have been shown to have a substantial survival advantage and novel treatment strategies are urgently needed. Thermal and laser based ablative techniques are widely used in many solid organ malignancies. Initial studies in the pancreas were associated with significant morbidity and mortality, which limited widespread adoption. Modifications to the various applications, in particular combining the techniques with high quality imaging such as computed tomography and intraoperative or endoscopic ultrasound has enabled real time treatment monitoring and significant improvements in safety. We conducted a systematic review of the literature up to October 2013. Initial studies suggest that ablative therapies may confer an additional survival benefit over best supportive care but randomised studies are required to validate these findings.

Key words: Pancreatic cancer; Radiofrequency ablation; Photodynamic therapy; Cryoablation; Microwave ablation; High frequency focused ultrasound; Irreversible electroporation

Core tip: Unresectable locally advanced pancreatic cancer with or without metastatic disease is associated with a very poor prognosis. Current standard therapy is limited to chemotherapy or chemoradiotherapy. Few regimens have been shown to have a substantial survival advantage and novel treatment strategies are urgently needed. Initial studies of ablation in the pancreas were associated with significant morbidity and mortality, which limited widespread adoption. Modifications to the various applications, in particular combining the techniques with high quality imaging such as computed tomography and intraoperative or endoscopic ultrasound has enabled real time treatment monitoring and significant improvements in safety.

BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) is the tenth most common cancer in the UK but the fifth commonest

Table 1 Use of ablative therapies to treat cystic and solid premalignant lesions of the pancreas

Author	Premalignant lesion	*n*	Treatment	Median area of ablation, mm (range)	Outcome	Complications
Gan *et al*[46]	Cystic tumours of the pancreas	25	EUS guided ethanol lavage	19.4 (6-30)	Complete resolution 35%	None
Oh *et al*[73]	Cystic tumours of the pancreas	14	EUS guided ethanol lavage + paclitaxel	25.5 (17-52)	Complete resolution in 79%	Acute pancreatitis (*n* = 1) Hyperamylasaemia (*n* = 6) Abdominal pain (*n* = 1)
Oh *et al*[74]	Cystic tumours of the pancreas	10	EUS guided ethanol lavage + paclitaxel	29.5 (20-68)	Complete resolution in 60%	Mild pancreatitis (*n* = 1)
DeWitt *et al*[75]	Cystic tumours of the pancreas	42	Randomised double blind study: Saline *vs* ethanol	22.4 (10-58)	Complete resolution in 33%	Abdominal pain at 7 d (*n* = 5) Pancreatitis (*n* = 1) Acystic bleeding (*n* = 1)
Oh *et al*[47]	Cystic tumours of the pancreas	52	EUS guided ethanol lavage + paclitaxel	31.8 (17-68)	Complete resolution in 62%	Fever (1/52) Mild abdominal discomfort (1/52) Mild pancreatitis (1/52) Splenic vein obliteration (1/52)
Levy *et al*[76]	PNET	8	EUS guided ethanol lavage (5 patients) and intra-operative ultrasound guided (IOUS) ethanol lavage (3 patients)	16.6 (8-21)	Hypoglycemia symptoms disappeared 5/8 and significantly improved 3/8	EUS guided: No complications. IOUS-guided ethanol injection: Minor peritumoral bleeding (1/3), pseudocyst (1/3), pancreatitis (1/3)
Pai *et al*[21]	Cystic tumours of the pancreas + neuroendocrine tumours	8	EUS guided RFA	Mean size pre RFA, 38.8 mm *vs* mean size post RFA, 20 mm	Complete ablation in 25% (2/8)	2/8 patients had mild abdominal pain that resolved in 3 d

RFA: Radiofrequency ablation; EUS: Endoscopic ultrasound; PNET: Pancreatic neuroendocrine tumour.

Table 2 Endoscopic ultrasound administered non-ablative anti-tumour therapies for pancreatic ductal adenocarcinoma

Author	Therapy	Patients	*n*	Outcome and survival	Complications
Chang *et al*[77]	Cytoimplant (mixed lymphocyte culture)	Unresectable PDAC	8	Median survival: 13.2 mo. 2 partial responders and 1 minor response	7/8 developed low-grade fever 3/8 required biliary stent placement
Hecht *et al*[78]	ONYX-015 (55-kDa gene-deleted adenovirus) + IV gemcitabine	Unresectable PDAC	21	No patient showed tumour regression at day 35. After commencement of gemcitabine, 2/15 had a partial response	Sepsis: 2/15 Duodenal perforation: 2/15
Hecht *et al*[79] Chang *et al*[80,81]	TNFerade (replication-deficient adenovector containing human tumour necrosis factor (TNF)-α gene)	Locally advanced PDAC	50	Response: One complete response, 3 partial responses. Seven patients eventually went to surgery, 6 had clear margins and 3 survived > 24 mo	Dose-limiting toxicities of pancreatitis and cholangitis were observed in 3/50
Herman *et al*[82]	Phase III study of standard care plus TNFerade (SOC + TNFerade) *vs* standard care alone (SOC)	Locally advanced PDAC	304 (187 SOC + TNFerade)	Median survival: 10.0 mo for patients in both the SOC + TNFerade and SOC arms [hazard ratio (HR), 0.90, 95%CI: 0.66-1.22, *P* = 0.26]	No major complications. Patients in the SOC + TNFerade arm experienced more grade 1 to 2 fever than those in the SOC alone arm (*P* < 0.001)
Sun *et al*[83]	EUS-guided implantation of radioactive seeds (iodine-125)	Unresectable PDAC	15	Tumour response: "partial" in 27% and "minimal" in 20%. Pain relief: 30%	Local complications (pancreatitis and pseudocyst formation) 3/15. Grade III hematologic toxicity in 3/15
Jin *et al*[84]	EUS-guided implantation of radioactive seeds (iodine-125)	Unresectable PDAC	22	Tumour response: "partial" in 3/22 (13.6%)	No complications

PDAC: Pancreatic ductal adenocarcinoma; EUS: Endoscopic ultrasound.

the zone of ablation such as the normal pancreas, duodenum, biliary tree or peri-pancreatic vasculature. These early studies applied high temperatures (> 90 ℃) and multiple rounds of ablation to treat large tumours in the head of the pancreas in one session[13]. An *ex-vivo* study of the thermal kinetic characteristics of RFA found that the optimal settings for RFA in the pancreas to prevent injury to the adjacent viscera was 90 ℃ applied for 5 min[15]. Subsequent clinical studies that reduced the RFA temperature from 105 ℃ to 90 ℃, reported only minimal RFA-related complications[7]. Active cooling of the major vessels and duodenum with saline during intraoperative RFA and observing at least a 0.5 cm area between the zone of ablation and major structures, reduced compli-

Duplicate articles, review articles and non-English publications which were excluded *n* = 10946

Studies included in the analysis
n = 32
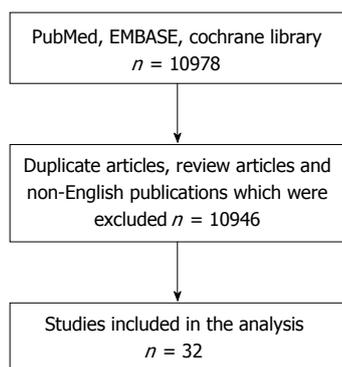
Figure 1 Systematic review schema.

cations[10,16,17]. Since most of the mortality resulted from uncontrollable gastrointestinal haemorrhage from ablated tumours in the head of the pancreas, some authors have recommended this probe should only be employed in body or tail tumours[10,16].

All studies have demonstrated that RFA leads to tumour necrosis and a decrease of tumour volume[9,12,17,18]. Some studies have also observed an improvement in tumour related symptoms, in particular a reduction of back pain and analgesia requirements. Tumour markers (carbohydrate antigen 19-9) also decrease following effective ablation[16]. Although all patients treated with RFA ultimately developed disease progression[9,11,12,17,18], when compared to patients with advanced disease who received standard therapy in a non-randomised cohort study, patients who received combination therapy had prolonged survival (33 mo *vs* 13 mo, *P* = 0.0048)[11]. However, this was a single centre study that only included 25 patients (12 receiving RFA). An earlier non-randomised study did not demonstrate the same survival advantage[12]. Spiliotis *et al*[11] also evaluated overall survival following RFA according to tumour stage. Patients with stage Ⅲ disease had a significant improvement in survival following RFA compared to patients with the same stage of disease receiving best supportive care (*P* = 0.0032). In contrast, no difference in overall survival was shown in patients with metastatic PDAC, following RFA treatment (*P* = 0.1095). Larger studies, in combination with systemic chemotherapy, would be needed to evaluate any potential role of RFA in patients with metastatic disease.

Recently two new RFA probes have been developed that can be placed down the working channel of an endoscope, enabling RFA to be administered under EUS guidance. Twenty-two patients with locally advanced PDAC were treated with the cryotherm probe (CTP) (ERBE Elektromedizin GmbH, Tübingen, Germany) that incorporates radiofrequency ablation with cryogenic cooling. The probe was sited successfully in 16 patients (72.8%); stiffness of the gastrointestinal wall and tumour prevented placement in the others. Following the procedure three patients reported mild abdominal pain and one experienced minor gastrointestinal bleeding, not re-

quiring transfusion[19]. In a further study, 7 patients with unresectable PDAC received EUS guided RFA using the monopolar radiofrequency (RF) catheter (1.2 mm Habib EUS-RFA catheter, Emcision Ltd, London). The tumour was shown to decrease in size in all cases and only one patient developed mild pancreatitis[20]. Long-term follow up is not available on the efficacy of these new catheters. Early clinical studies have also used the Habib EUS RFA catheter to treat cystic tumours of the pancreas (Table 1)[21].

Microwave ablation

Microwave (MW) current is produced by a generator connected *via* a coaxial cable to 14-gauge straight MW antennas with a 3.7 cm or 2 cm radiating section. One or two antennae are then inserted into the tumour for 10 min. The largest case series of microwave ablation in locally advanced PDAC includes 15 patients. Although MW ablation can be performed percutaneously or intraoperatively[22], in this series it was performed intraoperatively at the time of palliative bypass surgery. All tumours were located in the head or body of the pancreas and had an average size of 6 cm (range 4-8 cm); none had distant metastasis on imaging. Partial necrosis was achieved in all patients and there was no major procedure-related morbidity or mortality. However minor complications were seen in 40% (mild pancreatitis, asymptomatic hyperamylasia, pancreatic ascites, and minor bleeding). The longest survival of an individual patient in this series was 22 mo[23].

Cryoablation

The successful use of cryoablation in the pancreas was first reported in primate experiments in the 1970s[24]. However its potential application as a therapy in pancreatic cancer was not described for a further 20 years[25]. Cryoablation is most commonly performed intraoperatively under ultrasound guidance. Small lesions (< 3 cm) can be reliably frozen with a single, centrally placed probe but larger tumours require the placement of multiple probes or sequential treatments. Most studies have used the argon-gas-based cryosurgical unit (Endocare, Inc., CA, United States) and employ a double "freeze/thaw" cycle. The tumour is cooled to -160 ℃ and the resulting iceball monitored with ultrasound to ensure the frozen region encompasses the entire mass and does not compromise local structures. The tissue is then allowed to slowly thaw to 0 ℃ and a second cycle of freezing is performed after any necessary repositioning of the cryoprobes. Like in many of the RFA studies, the authors advocated a 0.5 cm margin of safety from major structures and that ideally the procedure should be performed at the same time as palliative bypass surgery or endoscopic biliary and duodenal stenting. Ablation of liver metastases can also be performed simultaneously[26].

The largest experience of intraoperative and percutaneous cryoablation in pancreatic cancer has been reported from Asia. To date more than 200 patients with

Table 3 Studies of radiofrequency ablation in pancreatic ductal adenocarcinoma

Study	Patients	n	Route of administration	Device	RFA temp (°C)	RFA duration (min)	Outcome	Complications
Matsui et al[12]	Unresectable PDAC	20 LA:9 M:11	At laparotomy 4 RFA probes were inserted into the tumour 2 cm apart	A 13.56-MHz RFA pulse was produced by the heating apparatus	50	15	Survival: 3 mo	Mortality: 10% (septic shock and gastrointestinal bleeding)
Hadjicostas et al[14]	Locally advanced and unresectable PDAC	4	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	NR	2-8	All patients were alive one year post-RFA	No complications encountered
Wu et al[10]	Unresectable PDAC	16 LA:11 M:5	Intraoperative	Cool-tip™ RFAblation system	30-90	12 at 30°C then 1 at 90°C	Pain relief: back pain improved (6/12)	Mortality: 25% (4/16) Pancreatic fistula: 18.8% (3/16)
Spiliotis et al[11]	Stage III and IV PDAC receiving palliative therapy	12 LA:8 M:4	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-7	Mean survival: 33 mo	Morbidity: 16% (biliary leak) Mortality: 0%
Girelli et al[7]	Unresectable locally advanced PDAC	50	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	105 (25 pts) 90 (25 pts)	Not reported	Not reported	Morbidity 40% in the first 25 patients. Probe temperature decreased from 105°C to 90°C Morbidity 8% in second cohort of 25 patients. 30-d mortality: 2%
Girelli et al[50]	Unresectable locally advanced PDAC	100	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-10	Median overall survival: 20 mo	Morbidity: 15%. Mortality: 3%
Giardino et al[51]	Unresectable PDAC. 47 RFA alone. 60 had RFA + radiochemotherapy (RCT) and/or intra-arterial systemic chemotherapy (IASC)	107	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-10	Median overall survival: 14.7 mo in RFA alone but 25.6 mo in those receiving RFA + RCT and/or IADC (P = 0.004)	Mortality: 1.8% (liver failure and duodenal perforation) Morbidity: 28%
Arcidiacono et al[39]	Locally advanced PDAC	22	EUS-guided	Cryotherm probe; bipolar RFA + cryogenic cooling	NR	2-15	Feasible in 16/22 (72.8%)	Pain (3/22)
Steel et al[41]	Unresectable malignant bile duct obstruction (16/22 due to PDAC)	22	RFA + SEMS placement at ERCP	Habib EndoHPB wire guided catheter	NR	Sequential 2 min treatments - median 2 (range 1-4)	Median survival: 6 mo Successful biliary decompression (21/22)	Minor bleeding (1/22) Asymptomatic biochemical pancreatitis (1/22), percutaneous gallbladder drainage (2/22). At 90-d, 2/22 had died, one with a patent SEMS
Figueroa-Barojas et al[42]	Unresectable malignant bile duct obstruction (7/20 due to PDAC)	20	RFA + SEMS placement at ERCP	Habib EndoHPB wire guided catheter	NR	Sequential 2 min treatments	SEMS occlusion at 90 d (3/22) Bile duct diameter increased by 3.5mm post RFA (P = 0.0001)	Abdominal pain (5/20), mild post-ERCP pancreatitis and cholecystitis (1/20)
Pai et al[20]	Locally advanced PDAC	7	EUS-guided	Habib EUS-RFA catheter	NR	Sequential 90s treatments - median 3 (range 2-4)	2/7 tumours decreased in size	Mild pancreatitis: (1/7)

PDAC: Pancreatic ductal adenocarcinoma; LA: Locally advanced PDAC; M: Metastatic PDAC; SEMS: Self-expanding metal stent; RFA: Radiofrequency ablation; EUS: Endoscopic ultrasound; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 4 Studies of cryoablation in pancreatic ductal adenocarcinoma

Study	*n*	Patients	Study	Outcome	Complications
Patiutko *et al*[25] (non-English article)	30	Locally advanced PDAC	Combination of cryosurgery and radiation	Pain relief and improvement in performance status: 30/30	Not reported
Kovach *et al*[52]	9	Unresectable PDAC	Phase I study of intraoperative cryoablation under US guidance. Four had concurrent gastrojejunostomy	7/9 discharged with non-intravenous analgesia and 1/9 discharged with no analgesia	No complications reported
Li *et al*[53] (non-English article)	44	Unresectable PDAC	Intraoperative cryoablation under US guidance	Median overall survival: 14 mo	40.9% (18/44) had delayed gastric empting. 6.8% (3/44) had a bile and pancreatic leak
Wu *et al*[54] (non-English article)	15	Unresectable PDAC	Intraoperative cryoablation under US guidance	Median overall survival: 13.4 mo	1/15 patients developed a bile leak
Yi *et al*[55] (non-English article)	8	Unresectable PDAC	Intraoperative cryoablation under US guidance	Not reported	25% (2/8) developed delayed gastric emptying
Xu *et al*[26]	38	Locally advanced PDAC, 8 had liver metastases	Intraoperative or percutaneous cryoablation under US or CT guidance + (125) iodine seed implantation	Median overall survival: 12 mo. 19/38 (50.0%) survived more than 12 mo	Acute pancreatitis: 5/38 (one has severe pancreatitis)
Xu *et al*[56]	49	Locally advanced PDAC, 12 had liver metastases	Intraoperative or percutaneous cryoablation under US or CT guidance and (125) iodine seed implantation. Some patients also received regional celiac artery chemotherapy	Median survival: 16.2 mo. 26 patients (53.1%) survived more than 12 mo	Acute pancreatitis: 6/49 (one had severe pancreatitis)
Li *et al*[57]	68	Unresectable PDAC requiring palliative bypass	Retrospective case-series of intraoperative cryoablation under US guidance, followed by palliative bypass	Median overall survival: 30.4 mo (range 6-49 mo)	Postoperative morbidity: 42.9%. Delayed gastric emptying occurred in 35.7%
Xu *et al*[58]	59	Unresectable PDAC	Intraoperative or percutaneous cryotherapy	Median survival: 8.4 mo. Overall survival at 12 mo: 34.5%	Mild abdominal pain: 45/59 (76.3%) Major complications (bleeding, pancreatic leak): 3/59 (5%) 1/59 developed a tract metastasis
Niu *et al*[29]	36 (CT) 31 (CIT)	Metastatic PDAC	Intraoperative cryotherapy (CT) or cryoimmunotherapy (CIT) under US guidance	Median overall survival in CIT: 13 mo CT: 7 mo	Not reported

PDAC: Pancreatic ductal adenocarcinoma.

unresectable pancreatic cancer have undergone cryoablation alone or in combination with other therapies (Table 4). Effective control of pain, normalisation of CA 19-9, improvement in performance status and prolonged survival have all been reported following cryoablation. Rates of significant complications appear to be lower than in other methods of ablation. Although some patients did encounter delayed gastric emptying following the treatment, this commonly settled with conservative management within a few days. Studies to date are summarised in Table 5. The process has also been shown to initiate antiangiogenesis and a systemic immunological response, which may promote additional anti-tumour effects[27,28]. However evaluation through larger studies will be necessary to fully determine this effect.

Early clinical studies have also combined the administration of cryotherapy with immunotherapy. In a study of 106 patients with unresectable PDAC, 31 received cryoimmunotherapy, 36 cryotherapy, 17 immunotherapy and 22 chemotherapy. Median overall survival was higher in the cryoimmunotherapy (13 mo) and cryotherapy groups (7 mo) than in the chemotherapy group (3.5 mo; both $P < 0.001$) and was higher in the cryoimmunotherapy group than in the cryotherapy ($P < 0.05$) and immunotherapy groups (5 mo; $P < 0.001$)[29].

LASER BASED ABLATIVE THERAPY

Photodynamic therapy

Photodynamic therapy (PDT) results in tumour ablation by exposure to light following an intravenous injection of a photosensitiser [*e.g.*, *meso*-tetra(hydroxyphenyl)chlorin (mTHPC), porfimer sodium or verteporfin] which is taken up by cells. It leads to a predictable zone of ablation within the tumour. To date, light has been delivered *via* small optic fibers which have nearly always been positioned percutaneously under image guidance (*e.g.*, CT)[30-32]. However these fibers can pass through a 19G needle, so administration under endoscopic ultrasound guidance is feasible.

The first Phase I trial of PDT in locally advanced PDAC was conducted in 2002. Substantial tumour necro-

Table 5 Studies of photodynamic therapy in pancreatic ductal adenocarcinoma

Study	*n*	Study	Photosensitiser	Number of fibres	Number of ablations	Outcome and survival	Complications
Bown *et al*[30]	16	CT guided percutaneous PDT to locally advanced but inoperable PDAC without metastatic disease	mTH-PC	Single	1	Tumour necrosis: 16/16. Median survival: 9.5 mo. 44% (7/16) survived > 1 year	Significant gastrointestinal bleeding: 2/16 (controlled without surgery)
Huggett *et al*[31,32]	13 + 2	CT guided percutaneous PDT to locally advanced but inoperable PDAC without metastatic disease	Verteporfrin	Single (13) Multiple (2)	1	Technically feasible: 15/15. Dose dependent necrosis occurred	Single fibre: No complications. Multiple fibres: CT evidence of inflammatory change anterior to the pancreas, no clinical sequelae

PDAC: Pancreatic ductal adenocarcinoma; CT: Computed tomography.

sis was achieved in all 16 patients included in the study. Median survival after PDT was 9.5 mo (range 4-30 mo). 44% (7/16) were alive one year after PDT. Two of the patients who had a pancreatic tumor which involved the gastroduodenal artery developed significant gastrointestinal bleeding following the procedure. However both were managed endoscopically with transfusion, without the need for surgery[30].

A significant drawback of the early PDT treatments was that patients had to spend several days in subdued lighting following the treatment to prevent complications from skin necrosis. However, newer photosensitisers with a shorter drug-light interval and faster drug elimination time have been developed (*e.g.,* verteporfrin) and have been shown in preclinical and early clinical studies to have a similar efficacy and safety profile to mTHPC[33]. A Phase I study by our group evaluated verteporfin-mediated PDT in 15 patients with unresectable locally advanced pancreatic cancer (Vertpac-01) (Table 5)[31,32]. The study was designed in 2 parts: the first 13 patients were treated with a single-fibre, with the following 2 patients being treated with light from multiple fibers. A predictable zone of necrosis surrounding the fibers was achieved. No instances of photosensitivity were reported and only one patient developed cholangitis. Patient went on to receive palliative gemcitabine chemotherapy 28 d after ablation.

YAG Laser
The neodymium-doped yttrium aluminium garnet (Nd: YAG) laser has been used to ablate pancreatic tumours in animal models[34]. A well demarcated area of necrosis and no complications were achieved, suggesting the potential for this therapy, but to date there have been no clinical studies.

NON-THERMAL, NON-LASER METHODS OF ABLATION
Many of the studies of thermal and light ablation techniques in locally advanced and metastatic PDAC have sug-

gested that cytoreduction may improve survival. However in the initial clinical studies some of the techniques were associated with unacceptably high rates of complications. This has led to a search for non-thermal alternative ablative therapies for use in PDAC.

High-intensity focused ultrasound
High intensity focused ultrasound (HIFU) therapy is a non-invasive method of ablation. Ultrasound energy from an extracorporeal source is focused on the pancreatic tumour to induce thermal denaturation of tissue without affecting surrounding organs[35]. Multiple non-randomised studies and case series, largely from Asia, have reported preliminary clinical experiences of using HIFU in PDAC. They have demonstrated that the technique is able to achieve tumour necrosis with relatively few side effects (Table 6). Recently a HIFU transducer has been designed which can be attached to an EUS scope to deliver HIFU locally to pancreatic tumours, thus preventing occasional burns to the skin. Initial animal studies have demonstrated that it can successfully abate the normal pancreas and liver[36].

Irreversible electroporation
NanoKnife® (Angiodynamics, Inc., NY, United States) or irreversible electroporation (IRE) is an emerging non-thermal ablative technique which uses electrodes, placed in the tumour, to deliver up to 3 kV of direct current. This induces the formation of nanoscale pores within the cell membrane of the targeted tissue, which irreversibly damages the cell's homeostatic mechanism, causing apoptosis. The United States Food and Drug Administration have recently approved the technique for use in the pancreas.

One of the major advantages of this technique is that it can be used in tumours that are in close proximity to peri-pancreatic vessels without risk of vascular trauma. The largest series of percutaneous IRE in PDAC includes 14 patients who had unresectable tumours and were not candidates for, or were intolerant of standard therapy[37]. The procedure was performed under general anaesthesia with complete muscle paralysis. Two patients

Table 6 Studies of high intensity focused ultrasound in pancreatic ductal adenocarcinoma

Study	n	Study	Outcome and survival	Complications
Wang *et al*[59] (non-English article)	15	HIFU monotherapy in late stage PDAC	Pain relief: 13/13 (100%)	Mild abdominal pain (2/15)
Xie *et al*[60] (non-English article)	41	HIFU alone *vs* HIFU + gemcitabine in locally advanced PDAC	Pain relief: HIFU (66.7%), HIFU + gemcitabine (76.6%)	None
Xu *et al*[61] (non-English article)	37	HIFU monotherapy in advanced PDAC	Pain relief: 24/30 (80%)	None
Yuan *et al*[62] (non-English article)	40	HIFU monotherapy	Pain relief: 32/40 (80%)	None
Wu *et al*[63]	8	HIFU in advanced PDAC	Median survival: 11.25 mo Pain relief: 8/8	None
Xiong *et al*[64]	89	HIFU in unresectable PDAC	Median survival: 26.0 mo (stage II), 11.2 mo (stage III) and 5.4 mo (stage IV)	Superficial skin burns (3.4%), subcutaneous fat sclerosis (6.7%), asymptomatic pseudocyst (1.1%)
Zhao *et al*[65]	37	Phase II study of gemcitabine + HIFU in locally advanced PDAC	Overall survival: 12.6 mo (95%CI: 10.2-15.0 mo) Pain relief: 78.6%	16.2% experienced grade 3 or 4 neutropenia, 5.4% developed grade 3 thrombocytopenia, 8% had nausea vomiting
Orsi *et al*[66]	6	HIFU in unresectable PDAC	Pain relief: 6/6 (100%)	Portal vein thrombosis (1/6)
Sung *et al*[67]	46	Stage III or IV PDAC	Median survival: 12.4 mo. Overall survival at 12 mo was 30.4%	Minor complications (abdominal pain, fever and nausea): 57.1% (28/29) Major complications (pancreaticoduodenal fistula, gastric ulcer or skin burns): 10.2% (5/49)
Wang *et al*[68]	40	Advanced PDAC	Median overall survival: 10 mo (stage III) and 6 mo (stage IV). Pain relief: 35/40 (87.5%)	None
Lee *et al*[69]	12	HIFU monotherapy in unresectable PDAC (3/12 received chemotherapy)	Median overall survival for those receiving HIFU alone (9/12 patients): 10.3 mo	Pancreatitis: 1/12
Li *et al*[70]	25	Unresectable PDAC	Median overall survival: 10 mo. 42% survived more than 1 year. Performance status and pain levels improved: 23/25	1st degree skin burn: 12% Mortality: 0%
Wang *et al*[71]	224	Advanced PDAC	Not reported	Abdominal distension, anorexia and nausea: 10/ 224 (4.5%). Asymptomatic vertebral injury: 2/224
Gao *et al*[72]	39	Locally advanced PDAC	Pain relief: 79.5% Median overall survival: 11 mo. 30.8% survived more than one year	None

HIFU: High intensity focused ultrasound; PDAC: Pancreatic ductal adenocarcinoma.

subsequently underwent surgery after IRE and both had margin-negative resections; both remain disease-free after 11 and 14 mo, respectively. Complications included spontaneous pneumothorax during anaesthesia (*n* = 1) and pancreatitis (*n* = 1); both patients recovered completely. No deaths were related to the procedure but the three patients with metastatic disease subsequently died from disease progression.

COMBINING ABLATIVE THERAPIES WITH BILIARY STENTING

Tumours of the head of the pancreas commonly cause distal biliary obstruction, which is managed in most cases by an endoscopically inserted self-expanding metal stent (SEMS). However due to tumour ingrowth SEMS are associated with a shorter patency time than bypass surgery. Hence there has been a growth in interest in using ablative therapies such as PDT or RFA to prolong stent

patency or to unblock a SEMS, which is already *in situ*. Randomised studies comparing PDT with biliary stenting to stenting alone have had conflicting results. Initial studies reported prolonged stent patency and improved survival after PDT[38,39]. However, a recent UK phase III study closed early as overall survival was longer in those treated with stenting alone[40]. The use of RFA in combination with SEMS placement has been reported in two small studies to date (Table 3). The investigators showed that the median bile duct diameter increased following endobiliary RFA and that 86% (19/22) of the SEMS were patent at 90 d[41,42]. Emerging evidence also suggests that endobiliary RFA may confer some early survival benefit in patients with malignant biliary obstruction independent of stent blockage and chemotherapy[41]. Occasionally centres have used RFA alone to achieve biliary drainage but results of on-going randomised controlled trials are awaited for validation of this technique[43]. Current guidance from the National Institute for Health and Care Excellence in the United Kingdom recommends

that this treatment should only be carried out in specialist centres in the context of clinical trials[44].

ENDOSCOPIC ULTRASOUND GUIDED NON-ABLATIVE LOCAL THERAPIES

Systemic chemotherapy agents are often associated with significant side effects, which can result in patients having to stop therapy or undergo dose reduction. Several groups have therefore explored using local anti-tumour agents in PDAC. The outcomes are summarised in Table 2.

PREMALIGNANT LESIONS OF THE PANCREAS

Some investigators have used similar ablative methods in PDAC to ablate premalignant solid and cystic lesions of the pancreas. Cystic lesions of the pancreas are an increasingly common clinical finding and some possess premalignant potential; longterm surveillance or surgery or pancreatic surgery is therefore recommended in accordance with international guidance[45]. Given the morbidity of surgery and uncertainties of surveillance for essentially benign disease, minimally invasive ablative therapies are increasingly becoming an attractive alternative treatment.

An EUS-guided injection of alcohol has been reported to have reasonable efficacy for achieving complete ablation of pancreatic cystic tumours (35%-62%). However, total cyst ablation was rare in septated cysts and the technique was associated with complications (pain and pancreatitis) in between 4%-20% of cases[46,47]. Occasional case reports have described using EUS guided alcohol injection to successfully ablate hepatic metastases[48] and pancreatic gastrointestinal stromal tumours[49]. Small case series have demonstrated EUS guided RFA can also be used safely for this indication[21]. Further validation will come from larger Phase Ⅱ studies.

CONCLUSION

Ablative therapies for unresectable pancreatic cancer are an attractive emerging therapy. All studies demonstrated that ablation is feasible and reproducible. Many of the early concerns that surrounded safety have been addressed with device development and modification of technique. Long-term survival data for many of the techniques is absent currently. Ultimately large prospective randomised studies will be required to assess the efficacy of these techniques and define their position in future treatment algorithms for the management of locally advanced pancreatic cancer.

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2014 ADVANCES IN PANCREATIC CANCER

Role of abnormal lipid metabolism in development, progression, diagnosis and therapy of pancreatic cancer

Julian Swierczynski, Areta Hebanowska, Tomasz Sledzinski

Julian Swierczynski, Areta Hebanowska, Department of Biochemistry, Medical University of Gdansk, 80-211 Gdansk, Poland

Tomasz Sledzinski, Department of Pharmaceutical Biochemistry, Medical University of Gdansk, 80-211 Gdańsk, Poland

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Correspondence to: Julian Swierczynski, Professor, Department of Biochemistry, Medical University of Gdansk, ul. Debinki 1, 80-211 Gdansk, Poland. juls@gumed.edu.pl

Telephone: +48-58-3491462   Fax: +48-58-3491465

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Abstract

There is growing evidence that metabolic alterations play an important role in cancer development and progression. The metabolism of cancer cells is reprogrammed in order to support their rapid proliferation. Elevated fatty acid synthesis is one of the most important aberrations of cancer cell metabolism. An enhancement of fatty acids synthesis is required both for carcinogenesis and cancer cell survival, as inhibition of key lipogenic enzymes slows down the growth of tumor cells and impairs their survival. Based on the data that serum fatty acid synthase (FASN), also known as oncoantigen 519, is elevated in patients with certain types of cancer, its serum level was proposed as a marker of neoplasia. This review aims to demonstrate the changes in lipid metabolism and other metabolic processes associated with lipid metabolism in pancreatic ductal adenocarcinoma (PDAC), the most common pancreatic neoplasm, characterized by high mortality. We also addressed the influence of some oncogenic factors and tumor suppressors on pancreatic cancer cell metabolism. Additionally the review discusses the potential role of elevated lipid synthesis in diagnosis and treatment of pancreatic cancer. In particular, FASN is a viable candidate for indicator of pathologic state, marker of neoplasia, as well as, pharmacological treatment target in pancreatic cancer. Recent research showed that, in addition to lipogenesis, certain cancer cells can use fatty acids from circulation, derived from diet (chylomicrons), synthesized in liver, or released from adipose tissue for their growth. Thus, the interactions between *de novo* lipogenesis and uptake of fatty acids from circulation by PDAC cells require further investigation.

Core tip: Metabolic alterations associated with mutation in oncogenes and tumor suppressor genes play an important role in cancer development and progression. One of the most important aberrations of metabolism in cancer cells is an elevated synthesis of lipids, which are building blocks for cell membrane formation during cell proliferation and signalling molecules. This review aims to demonstrate the changes in lipid metabolism in pancreatic ductal adenocarcinoma, the most common pancreatic neoplasm, with very high mortality. The potential role of elevated lipid synthesis in diagnosis, prognosis and therapy of pancreatic cancer is also discussed.

INTRODUCTION

Cancer development is generally attributed to the accumulation of genetic alterations, which leads to activation of cellular oncogenes and inactivation of tumor suppressor genes. Apart from mutations, epigenetic modulation, numerical and structural abnormalities in chromosomes, and aneuploidy are commonly observed in cancer cells, and may play a critical role in tumorigenesis[1]. In addition, carcinogenesis involves significant changes in cellular metabolism, especially in carbohydrate, lipid, nucleic acid, and amino acid metabolism (Figure 1).

The metabolism of cancer cells is reprogrammed in order to support their rapid proliferation. Nowadays, metabolic alteration, also referred to as metabolic transformation, should be added to six classic hallmarks of cancer cells proposed by Hanahan and Weinberg[2], Tennant *et al*[3] and illustrated on Figure 2. Over eight decades ago, Warburg revealed that an elevated rate of glycolysis under aerobic conditions, a phenomenon commonly known as the Warburg effect, is a distinctive feature of many human and animal tumors[4]. In the majority of cancers, glucose is converted mostly to lactate, and, therefore, only 2 moles of ATP per 1 mole of glucose are synthesized. In contrast, most non-cancer cells containing mitochondria, produce CO_2 and H_2O from glucose, and 38 moles of ATP are synthesized per 1 mole of glucose, under aerobic conditions.

Over the last two decades, several authors reported overexpression of genes encoding lipogenic enzymes in many human cancers (Table 1)[5-12]. This phenomenon is usually associated with an increased glucose carbon incorporation into lipids[13-16]. The possible pathways for the conversion of glucose into phospholipids and cholesterol, required for membrane formation in cancer cells, are illustrated on Figure 1. Pyruvate formed from glucose during active aerobic glycolysis, is either converted to lactate by lactate dehydrogenase (LDH), or can enter into mitochondria, where it is decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH). Then, by means of reactions of citrate synthase (CS), present in mitochondria, and ATP citrate lyase (ACLY), present in cytosol, cytosolic acetyl-CoA, a key substrate for lipid biosynthesis is formed (Figure 1). Elevated activities of both enzymes (CS and ACLY) are observed in some malignancies, and the inhibition of ACLY is known to lead to cessation of tumor growth[17-21]. Interestingly, some tumors display a diminished flux of glucose carbon through PDH-catalyzed reaction, due to increased PDHK (pyruvate dehydrogenase kinase) activity, under the influence either hypoxia or oncogenic factors. This points to the possible use of carbon source other than glucose, for lipid synthesis[22-25].

Through conversion to fructose 6-phosphate, glucose also serves as a substrate for hexosamine phosphate synthesis (according to reaction: fructose 6-phosphate + glutamine → glucosamine 6 phosphate + glutamate), required for biosynthesis of glycoproteins and glycosaminoglycans. Glucose may also be converted to pentose phosphate on pentose phosphate pathway (PPP), and then to phosphoribosyl pyrophosphate (PRPP), a precursor of purine and pyrimidine nucleotides necessary for DNA synthesis (Figure 1). PPP generates NADPH, which is required for many processes, including lipid biosynthesis (Figure 1). The activity of glucose 6-phosphate dehydrogenase (G6PDH), a rate limiting enzyme of PPP, is elevated in certain cancers, including human pancreatic cancer (PC)[19,26]. Glutamine for hexosamine and nucleotide synthesis may originate from citrate produced in mitochondria. Citrate is converted by Krebs cycle to 2-oxoglutarate, a precursor of glutamate (Figure 1), and later to glutamine. However, glutamine is not synthesized on that pathway in many cancer cells, but is rather taken up from the circulation, where it is one of the most abundant amino acids[27].

Glucose and glutamine are two main sources of energy and carbon for most cancer cells[28-30]. Some data suggest that glucose accounts mainly for lipid, purine, and pyrimidine nucleotide synthesis, whereas glutamine is contributing to: (1) anaplerotic re-feeding of Krebs cycle; (2) amino acid synthesis; and (3) providing nitrogen necessary for purine and pyrimidine nucleotide synthesis[14], however, there is also evidence of glutamine participation (as carbon donor) in lipid biosynthesis[31]. High expression of glutaminase-encoding gene was revealed during the S phase of the cell cycle in some cancer cell lines (*i.e.* HeLa cells), along with the low expression in G2/M phase[32]. Upon cellular uptake, glutamine is transported to mitochondria, and then converted to ammonia and glutamate by mitochondrial glutaminase. Then glutamate is deaminated to 2-oxoglutarate by glutamate dehydrogenase. In mitochondria, 2-oxoglutarate is further metabolized by Krebs cycle to malate (Figure 3). Part of the malate is released to cytosol, converted to pyruvate by NADP-linked malic enzyme (ME), and, finally, to lactate by LDH, similarly to pyruvate formed from glucose during glycolysis (Figure 3). The conversion of glutamine to lactate is called glutaminolysis analogically to glycolysis (Figure 3). The increased synthesis of lactic acid by cancer cells leads to the decrease in pH of tumor microenvironment, which promotes angiogenesis, invasion, and metastasis, and suppresses the anticancer immune response through diminished cytotoxic T-cell function[33] (Figure 3).

In a variety of tumors, pyruvate formed during active glutaminolysis is converted into acetyl-CoA by PDH (instead of being converted to lactate by LDH), and later to citrate, supplying carbons for lipid synthesis (Figure 3)[34,35]. Conversion of glutamine to citrate may be also the result of reductive carboxylation of 2-oxoglutarate
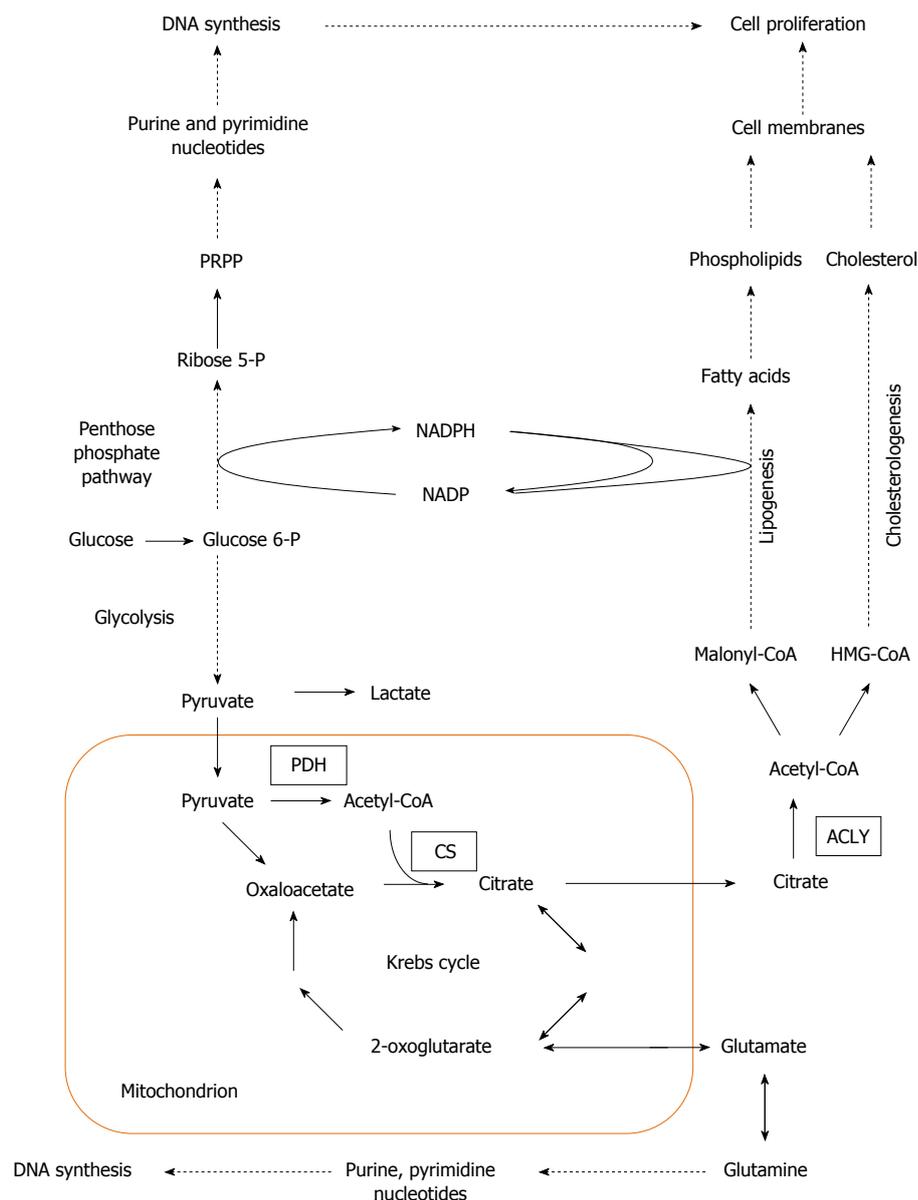
Figure 1 Cellular metabolism of cancer cells-association with cell proliferation. Solid arrows represent single reactions; dotted arrows represent processes including numerous reactions. PRPP: Phosphoribosyl pyrophosphate; Ribose 5-P: Ribose 5-phosphate; Glucose 6-P: Glucose 6-phosphate; PDH: Pyruvate dehydrogenase; CS: Citrate synthase; ACLY: ATP citrate lyase; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A.

derived from glutamine, catalyzed by two isoforms of NADP⁺-dependent isocitrate dehydrogenase - mitochondrial (IDH2), and/or cytosolic (IDH1) (Figure 1)[36-40]. In some cancer cell lines 10%-25% of fatty acids carbons are derived from glutamine under normoxia, and up to 80% under hypoxia[14,36,37]. Wise *et al*[38] suggest that IDH2 is mainly contributing to conversion of glutamine to lipids. However, other data show that in A549 (adenocarcinoma of human alveolar basal epithelial cells), and in renal carcinoma cells (RCC) cell lines IDH1 is more important[36]. In melanoma or osteosarcoma cell lines both IDH isoforms equally participate in 2-oxoglutarate reduction[37,40].

Continuous loss of citrate from mitochondria to cytosol requires replenishment of Krebs cycle intermediates. Glutamine serves as a key substrate for Krebs cycle intermediates in many cancer cells, and is critical for cell proliferation. A proliferating cell dies upon glutamine (but not glucose) withdrawal from the medium[41].

Fatty acid (FA) biosynthesis remains at a low level in most non-cancerogenic tissues, except liver and adipose tissue. The two latter lipogenic tissues convert the excess of carbohydrates to triacylglycerols[42-49]. Conversely FAs synthesized in cancer cells are esterified mainly to phospholipids required for membrane formation, which promotes cellular replication (Figure 1). Overall, coordinated enhancement of glucose, lipid, and amino acid metabolism, leading to increased synthesis of membrane lipids, nucleotides, and amino acids supports rapid proliferation of cancer cells (Figure 1).

Proliferation and metabolism of cancer cells share common regulatory pathways[50-53]. MYC, proto-oncogene and major regulator of transcription in growing cells, controls several metabolic processes such as: (1)
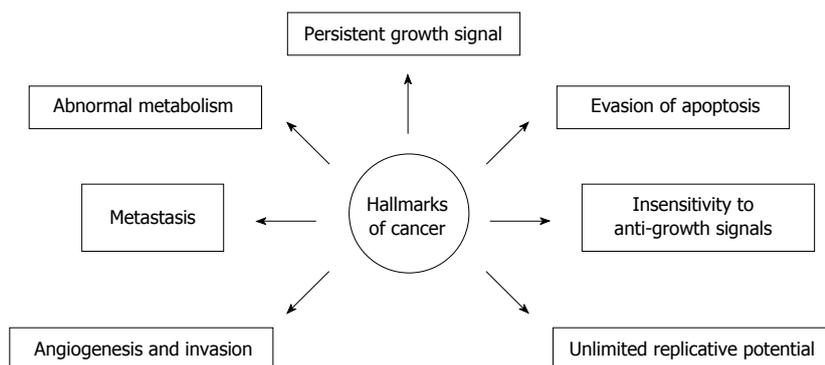
Figure 2 Hallmarks of cancer.

Table 1 Overexpression of lipogenic enzymes in human tumors

Enzyme name	Neoplasm type	Experimental model	Ref.
Fatty acid synthase	Pancreatic cancer	Human tumor tissue, cell line	[96,104,105]
(FASN)	Breast carcinoma	Human tumor tissue	[5,9,166]
	Prostate cancer	Human tumor tissue	[167]
	Melanoma	Human tumor tissue	[168]
	Nephroblastoma	Human tumor tissue	[169]
	Renal cancer	Cell line	[170]
	Endometrial carcinoma	Human tumor tissue	[12,171]
	Colon cancer	Human tumor tissue	[11,172]
	Ovarian neoplasms squamous cell	Human tumor tissue	[10,173]
	Carcinoma of the lung head and neck squamous	Human tumor tissue	[174]
	Cell carcinoma squamous cell	Human tumor tissue	[175]
	Carcinoma of the tongue	Human tumor tissue	[176]
ATP citrate lyase	Small cell lung cancer	Cell line	[251]
(ACLY)	Bladder cancer	Human tumor tissue	[7]
	Breast cancer	Cell line	[252]
	Gastric cancer	Human tumor tissue, cell line	[253]
	Colon cancer	Human tumor tissue	[254]
	Prostate cancer	Human tumor tissue	[254]
	Hepatocellular carcinoma	Human tumor tissue	[255]
Acetyl-CoA carboxylase	Prostate cancer	Human tumor tissue	[6]
(ACCA)	Hepatocellular carcinoma	Human tumor tissue	[255]
	Breast carcinoma	Human tumor tissue	[256]
Stearoyl-CoA desaturase	Pancreatic cancer	SCD1 indices in patients serum	[128]
(SCD1)	Clear cell renal cell carcinoma	Human tumor tissue	[200]
Acetyl-CoA synthetase	Colon adenocarcinoma	Human tumor tissue	[257]
(ACS)	Malignant glioma	Cell line	[258]
Citrate synthase	Pancreatic cancer	Human tumor tissue	[19]
(CS)	Renal cell carcinoma	Human tumor tissue	[20]

glycolysis and glutaminolysis; (2) nucleotide biosynthesis; and (3) lipid biosynthesis, and mitochondrial biogenesis[53]. Furthermore, MYC stimulates glutamine uptake and metabolism[54,55]. Tumor suppressor protein, p53, is involved in regulation of bioenergetic homeostasis and lipid metabolism in both normal and cancer cells[51,56-58]. p53 induces the expression mitochondrial glutaminase-encoding gene, increasing energy production from glutaminolysis[59,60]. Mutant p53 increases lipid synthesis, *via* sterol regulatory element-binding protein 1c (SREBP1c), and promotes ovarian cancer metastasis[52]. Certain oncoproteins such as: Akt, Ras, and Src, also stimulate glycolysis in transformed cells[50]. Regulation of glutamine metabolism by Rho GTPases and Ras was also proposed[61]. The oncogenes and tumor suppressor genes whose products participate in regulation of carbo-

hydrate, lipid, nucleotide and amino acid metabolism are presented in Table 2.

Also mutations of some genes can contribute to abnormal cellular metabolism, which in turn can affect oncogenic signaling pathways. For example mutation in gene encoding IDH1/2 is associated with deregulation of cellular metabolism, especially in glioma cells[62]. In glioma IDH1/2 mutations are responsible for conversion of 2-oxoglutarate to 2-hydroxyglutarate, which, by inhibition of 2-oxoglutarate-dependent dioxygenases, affects: (1) proto-oncogene expression; (2) DNA and histone modification; and (3) alteration of extracellular matrix proteins (due to inhibition of collagen hydroxylation)[62]. This paper reviews the possible role of lipid metabolism in human cancers, particularly in PC biology, prognosis, and treatment.
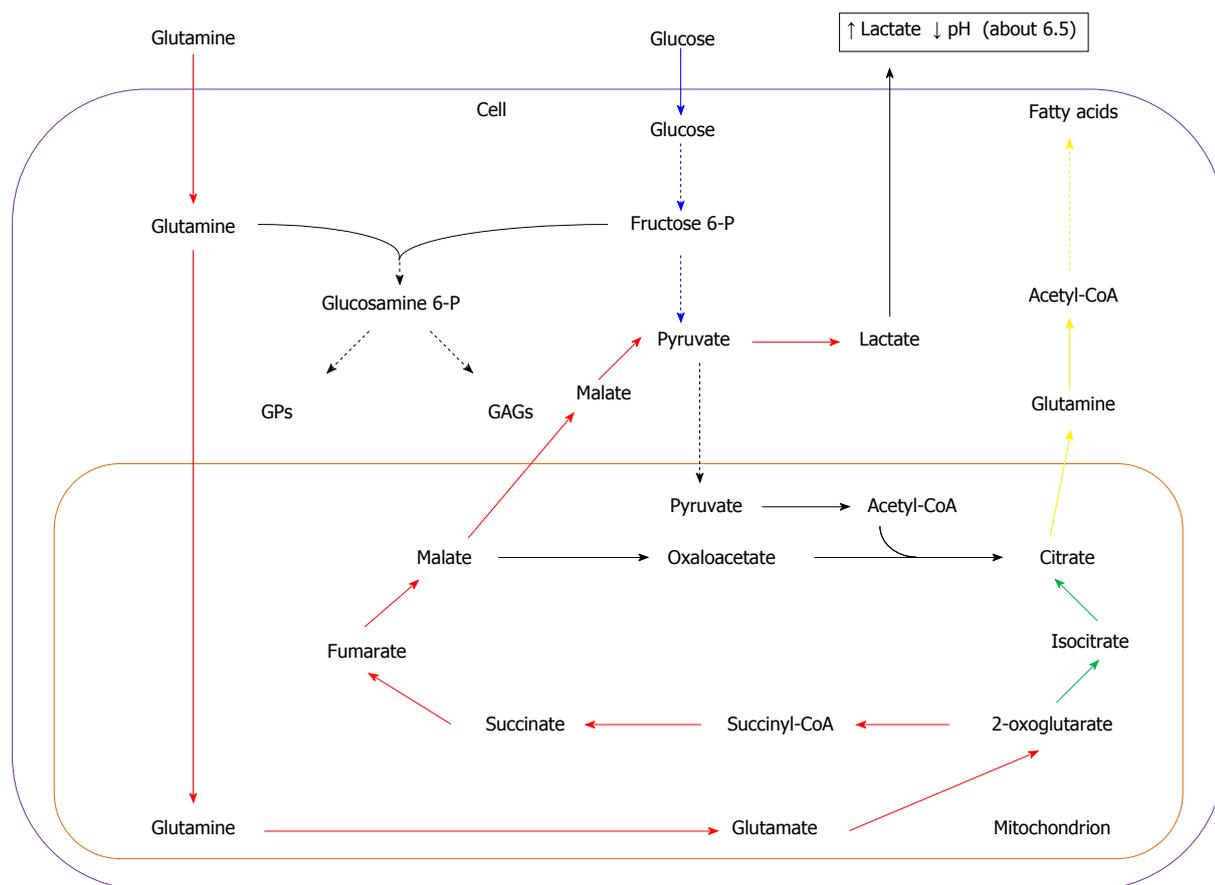
Figure 3 Glutamine metabolism of cancer cells. Red arrows represent glutaminolysis; green arrows represent "reversed Krebs cycle" reactions; blue arrows represent glycolysis; yellow arrows represent lipogenesis. Solid arrows represent single reactions; dotted arrows represent processes including numerous reactions. Fructose 6-P: Fructose 6-phosphate; GAGs: Glycosaminoglycans; GPs: Glycoproteins.

ABNORMAL LIPID METABOLISM IN PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic neoplasm, comprising approximately 90% of all pancreatic malignancies, and the eight leading cause of cancer-associated death in the world[63]. The 5-year survival rate of PDAC patients is approximately about 5%[64]. Surgery is the primary treatment modality and the only available chance for recovery, however only approximately 10% of patients are eligible for surgical treatment. Other therapies have proven ineffective thus far.

Similar to other cancers, both activation of oncogenes and inactivation of tumor suppressor genes play key role in PDAC pathogenesis. The most frequent genetic alterations documented in PCs, including PDAC, are presented in Table 3. Other pancreatic tumors show different aberrations (Table 4).

In addition to genetic and epigenetic alterations, development of PC involves significant alterations of cellular metabolism, supporting rapid proliferation of cancer cells. Reduced vascularity, leading to poor perfusion is characteristic for PC. This results in low availability of oxygen and nutrients[65,66]. The presence of hypoxia corresponds to highly aggressive character of PCs[67].

Oxygen deprivation of both non-cancer and cancer cells leads to the stabilization of hypoxia inducible factor 1α (HIF-1α), which dimerizes with HIF-1β, transfers into nucleus and binds with hypoxia-responsive elements present in DNA (Figure 4). This counteract the deleterious impact of decreased oxygen availability[68]. High level of HIF-1α is associated with increased glucose consumption due to activation of glucose transporter 1 (GLUT1), and glycolysis, especially hexokinase (1 and 2), and LDH[69-73] (Figure 4). Overexpression of HIF-1α in human PC cells makes this malignancy similar to other cancers[74]. Interestingly, the expression of HIF-1α in the hypoxic part of pancreatic tumor is at the same level as in its well-oxygenated fragments[75]. Some data indicate that phosphorylation of HIF-1α weakens the interaction of this protein with von Hippel-Lindau tumor suppressor (VHL) which normally stimulates degradation of HIF-1α during normoxia (Figure 4). The phosphorylation result from activation of MAPK or other protein kinase (putatively AKT) in cancer cells[76]. Both kinases are downstream effectors in various signaling pathways, including KRAS pathway. Continuous KRAS signaling and downstream activation of MAPK and AKT results from the mutation of KRAS (observed in 90% of PDACs), or can be stimulated by epidermal growth factor (EGF), prostaglandin E₂ (PGE₂), and some oxidants[73,77,78].

Table 2 Oncogenes and tumor suppressor genes, whose products participate in regulation of cancer cells metabolism

Oncogene/tumor suppressor	Metabolic pathway	Enzyme	Ref.
MYC	Glucose transport	GLUT1	[53-55]
	Glycolysis	Hexokinase 2	
		Phosphohexose isomerase	
		Phosphofructokinase 1	
		Aldolase A	
		3-phosphoglyceraldehyde dehydrogenase	
		Phosphoglycerate kinase	
		Phosphoglycerate mutase	
		Enolase 1	
		Pyruvate kinase 2	
		Lactate dehydrogenase A	
	Regulation of PDH	Pyruvate dehydrogenase kinase 1	
	Glutamine transport	Glutamine transporters ASCT2 and SN2	
	Glutaminolysis	Glutaminase 1	
	Serine hydroxymethyltransferase		
	Pyrimidine synthesis		
	Aminoacids metabolism	CAD	
		Ornithine decarboxylase	
	Lipogenesis	Fatty acid synthase	
p53	Glucose transport	GLUT1	[51,56-60]
	Glycolysis	Hexokinase 2	
		Fructose-2,6-bisphosphatase	
		Phosphoglycerate mutase	
	Oxidative phosphorylation	Cyrochrome c oxidase	
	Glutaminolysis	Glutaminase 2	
	Pentose Phosphate Pathway	Glucose-6-phosphate dehydrogenase	
	Regulation of PDH	Pyruvate dehydrogenase kinase 1	
	Krebs cycle	Aconitase	
KRAS	Glucose transport	GLUT1	[61,73,94]
	Glycolysis	Hexokinase 2	
		Phosphofructokinase 1	
		Lactate dehydrogenase A	
	Pentose phosphate pathway	Transketolase	
	Hexosamine synthesis	Phosphohexose aminotransferase	
	Glutaminolysis	Glutamate dehydrogenase	
		Aspartate transaminase	
Akt/PTEN	Glucose transport	GLUT1	[50,113-115]
	Lipogenesis	FASN	

PI3K/Akt signaling pathway leads to overexpression of HIF-1α, and directly participates in glucose transport and metabolism by regulating GLUT1 gene expression in PC cells, especially when the function of PTEN, tumor suppressor inhibiting PI3K/AKT pathway, is lost[79-81]. Another oncogene, MYC, interacts with KRAS and HIF-1α in PDAC metabolic switch. MYC response elements are present in most glycolytic genes, thus, allowing MYC protein to regulate glucose metabolism[73,82]. Some data suggest that activation of HIF-1α, leading to metabolic reprogramming of pancreatic cells during normoxia, is also controlled by β-adrenergic receptors through the transactivation of epidermal growth factor receptor (EGFR, requiring PKA activity), and further activation of AKT[83]. Also insulin, causing activation of PI3K/AKT and MAPK pathways, can be potential stimulator of HIF-1α activity acting independently of oxygen availability[84].

Mucin 1 (MUC1), a transmembrane protein involved in stabilization of HIF-1α is one of the newly discovered activators of HIF-1α in PC. Directly interacting with HIF-1α and DNA, MUC1 induces expression of glycolytic genes[85]. High activity of MUC1 is correlated with intensive growth and metastasis of pancreatic tumors[86,87]. HIF-1α is coexpressed with Nupr1 (also known as p8 or Com, i.e. candidate of metastasis) in human PDAC[88]. Nupr 1 is a chromatin protein, structurally related to the high-mobility group (HMG) protein, it interacts with several other proteins in the regulation of cell cycle, apoptosis, autophagy, and gene transcription[89]. It is responsible for increased resistance of stress-exposed PDAC cells[90] and supposedly interacts and amplify the KRAS signaling in cancer cells, in order to overcome the activity of some tumor suppressors (such as p16) action[91].

The data presented above suggest that several proteins (mainly products of proto-oncogens or tumor suppressor genes) might affect conversion of glucose to pyruvate in PDAC cells.

Most of the pyruvate formed as a result of increased glycolysis in PDAC cells, is metabolized to lactate, some pyruvate is used to citrate, and further to FAs biosynthesis[92,93]. Accordingly, the activity of CS, one of the crucial enzymes involved in pyruvate to FA conversion (Figure 1), is elevated in PC[19,20]. Thus, it is likely that citrate is synthesized from glucose in PC cells, although glutamine seems to play an important role as well.

Table 3 Oncogenes and tumor suppressor genes whose products alter the metabolism of pancreatic cancer cells

Gene	Protein	Mechanism of alteration in PDAC	Regulated processes in PDAC	Alteration in PDAC	Ref.
Oncogenes					
KRAS	KRAS	Point mutations	Cell proliferation and survival, motility, glucose transport, glycolysis, hexosamine synthesis, nonoxidative pentose phosphate pathway arm, glutaminolysis	> 95%	[73,94,259-261]
AKT	AKT	Mutations, amplification	Signal transduction, lipogenesis, glucose transport	10%-20%	[73,79-81,262-264]
c-erbB2	HER2	Overexpression amplification	Proliferation, differentiation, survival	20%-80%	[265-268]
Myc	MYC	Amplification overexpression	Glycolysis, glutaminolysis, PDH inhibition	70%	[55,73,82,94,269]
Tumor suppressor genes					
TP53	p53	Mutation and second allele deletion	Cell cycle, apoptosis, DNA repair, glucose transport, glycolysis, lipogenesis, ppp oxidative arm, glutaminolysis	50%-80%	[270-273]
Smad4/DPC4	SMAD4	Homozygous deletion, mutation and second allele deletion	Cell cycle, TGF-β signaling	55%	[274-276]
STK/LKB1	LKB1	Homozygous deletion, mutation and second allele deletion	Apoptosis, lipogenesis, energy production, protein synthesis	5%	[277-279]
CDKN2A/p16	p16	Homozygous deletion, mutation, hypermethylation	Cell cycle	95%	[280-282]
PTEN	PTEN	Hypermethylation, inhibition by miRNA	PI3K/AKT signaling pathway	30%-70%	[79,283,284]

PDAC: Pancreatic ductal adenocarcinoma.

Table 4 Most common genetic alterations observed in different types of human pancreatic cancers

Type of pancreatic cancer	Gene affected	Ref.
Pancreatic ductal adenocarcinoma (PDAC) (90% of all pancreatic cancers)	KRAS, AKT, MYC, TP53, SMAD4, CDKN2A, PTEN	[55,63,64,73,78,79,94,269,284-287]
Acinar cell carcinoma (ACCA) (< 1% of all pancreatic cancers)	APC/β-catenin (CTNNB1), BRCA2, BCL10	[288-290]
Adenosquamous carcinoma (ASC) (< 1% of all pancreatic cancers)	TP53, CDKN2A, KRAS, E-cadherin,	[291,292]
Intraductal papillary mucinous neoplasm (IPNM) (1%-3% of all pancreatic cancers)	GNAS, KRAS, RNF4, STK11/LKB1, MUC1, MUC2, hTERT, COX2, Shh	[278,293,294]
Mucinous cystic neoplasm (MCN) (< 1% of all pancreatic cancers)	KRAS, RNF4, TP53, CDKN2A	[295]
Serous cystadenoma (SCN) (< 1% of all pancreatic cancers)	VHL	[296]
Solid-pseudopapillary neoplasm (SPN) (1%-2% of all pancreatic cancers)	APC/β-catenin (CTNNB1), E-cadherin	[297,298]
Pancreatic neuroendocrine tumors (PanNET) (2%-5% of all pancreatic cancers)	DAXX, ATRX, MEN1, TSC2, PTEN, PI3KCA, CHGA, CHGB, mTOR	[299-302]

Son *et al*[94] suggested that KRAS directs glutamine carbons to Krebs cycle in PC cells, to export them to cytosol for cytosolic ME reaction. This results in the generation of NADPH, which is used for lipid biosynthesis and for redox state control. Deprivation of glutamine or inhibition of glutaminase activity are reflected by decreased production of ATP and higher levels of reactive oxygen species (ROS). Glutamine may also supply OAA, condensed with acetyl-CoA, to citrate synthesis, or be involved in citrate formation through reductive carboxylation of 2-oxoglutarate catalyzed by reverse IDH reaction. Although the involvement of glutamine was documented in some malignancies, its role in PC cells is still not completely understood[14,36-40,95]. Nevertheless, *de novo* biosynthesis of lipids (possibly from glucose and/or glutamine) is elevated in PDAC cells[96-98].

Gemcitabine, herceptin or irinotecan treatment has minimal impact on survival rates in patients with advanced PC[99,100]. In contrast treating PC patient with gemcitabine, α-lipoic acid, and hydroxycitrate yielded promising results[101]. Since hydroxycitrate is an inhibitor of ACLY, the activity of the latter lipogenic enzyme (splitting citrate to acetyl-CoA and OAA in cytosol) is likely elevated in PC cells as well, and, similar to other cancers, plays an important role in the development of this malignancy. The next stage of lipogenesis, leading to biosynthesis of malonyl-CoA (fatty acid synthase substrate), is catalyzed by acetyl-CoA carboxylase (ACCA). Phosphorylation by AMPK, leading to ACCA activity cessation, is one of the crucial stages of lipogenesis regulation in lipogenic tissues[102]. The activity of AMPK in PDAC cells is lower than in normal cells, mostly due to LKB1 tumor suppressor inhibition, leading to increased ACCA activity[103]. Fatty acid synthase (FASN) reaction constitutes the last step in palmitate synthesis. The significant role of FASN in cancer development was established approximately two
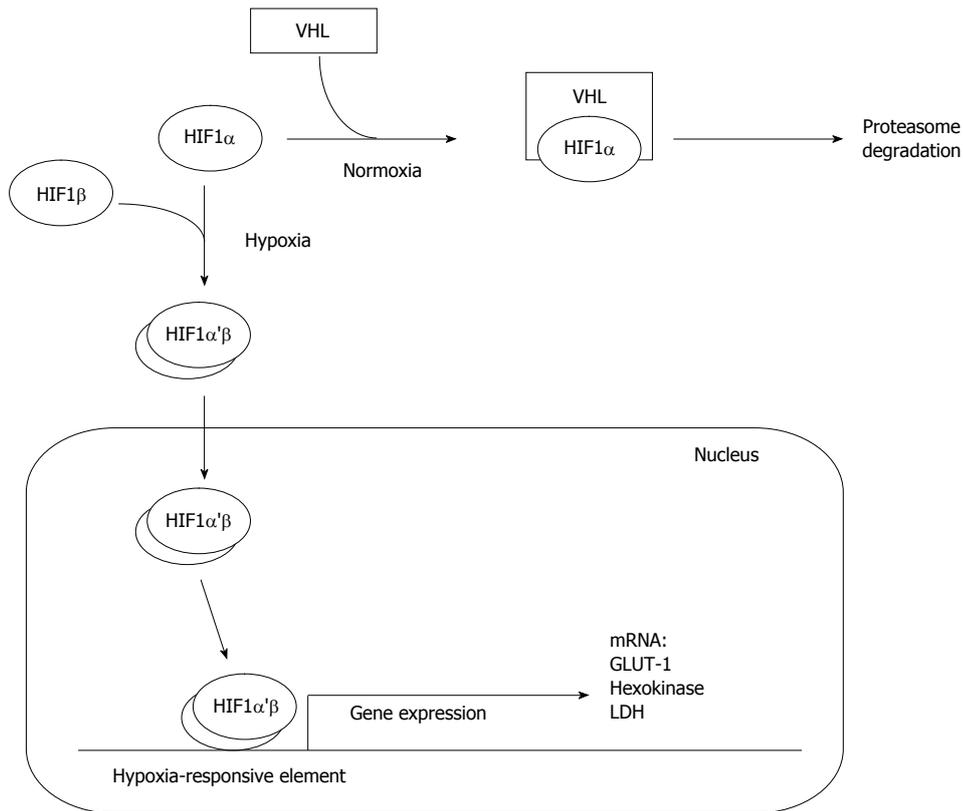
Figure 4 Role of hypoxia inducible factor 1 α'β in pancreatic cancer. HIF1α: Hypoxia inducible factor 1α; HIF1β: Hypoxia inducible factor 1β; VHL: Von Hippel-Lindau tumor suppressor; GLUT-1: Glucose transporter 1; LDH: Lactate dehydrogenase.

decades ago, when the oncogenic antigen-519 (OA-519), a molecular marker, was identified in breast cancer patients[9]. FASN utilizes acetyl-CoA (supplied by ACLY), malonyl-CoA (supplied by ACCA) and NADPH as a reducing equivalent. In the case of PC cells, NADPH is a product of PPP or reaction catalyzed by ME during oxidative decarboxylation of malate formed from glutamine (*i.e.* during glutaminolysis)[94]. FASN is the most extensively studied lipogenic enzyme in PDAC cells. Elevated expression of FASN-encoding gene was documented in human PC[96,104,105] and high level of FASN protein, both in tumor cells and in serum is associated with poor prognosis[96,98]. Furthermore inhibition of FASN activity was revealed to induce apoptosis in several tumors[106-110]. Indeed, FASN is an oncogenic protein and its overexpression in non-transformed human breast epithelial cells, can produce their cancer-like phenotype, in a HER1/2 dependent process[111]. Similar phenomenon was reported in the case of colorectal cancer cells[112]. The expression of *FASN* is strongly induced in hypoxia, by MAPK or PI3K/AKT signaling pathways. This results in activation of SREBP1c transcription factor, which directly binds to FASN promoter (and promoters of other lipogenic genes)[113,114]. Similar effect can be observed in the absence of PTEN tumor suppressor, which normally inhibits PI3K/AKT signaling[114,115]. Moreover, SREBP1c-independent regulation of *FASN*, mediated by HER2 with PI3K or mTOR involvement was observed in breast cancer cells[116]. Furthermore strong acidic environment

of breast cancer may promote epigenetic modification of *FASN* promoter, leading to increased expression of this gene[117]. As all those events take place in PC cells, the mechanism of *FASN* regulation in PDAC is probably similar as in the case of other malignancies.

Inhibited activity of FASN (or other lipogenic enzymes) is reflected by decreased tumor growth and may lead to apoptosis of some cancer cells. The inhibition of FASN was revealed to diminish proliferation of osteosarcoma and colorectal cancer cells, through decrease of HER2 activity, leading to down-regulation of PI3K/Akt signaling pathway[112,118]. Induction of apoptosis is likely to result from elevated concentration of malonyl-CoA, that is reflected by decreased oxidation of FA and increased ceramide concentration. Ceramide is a well-known activator of apoptosis, and its enhanced biosynthesis (along with inhibited ceramidase activity) leads to the death of PC cells[106,119]. Furthermore the altered composition of FAs in phospholipid structure (predominance of polyunsaturated acids over saturated and monounsaturated acids) increases the oxidative stress yielding the same result[120].

Glycolytic synthesis of ATP seems the most important pathway in hypoxic cancer cells. In the cases of normoxia, glucose is rather directed to PPP for NADPH and pentose synthesis, and KRAS acts as the main controlling factor supporting tumor cell proliferation[121,122]. Both oxidative and non-oxidative phases of PPP are upregulated in PC cells. The non-oxidative phase is upregulated by KRAS[73,123], whereas G6PDH activity (main
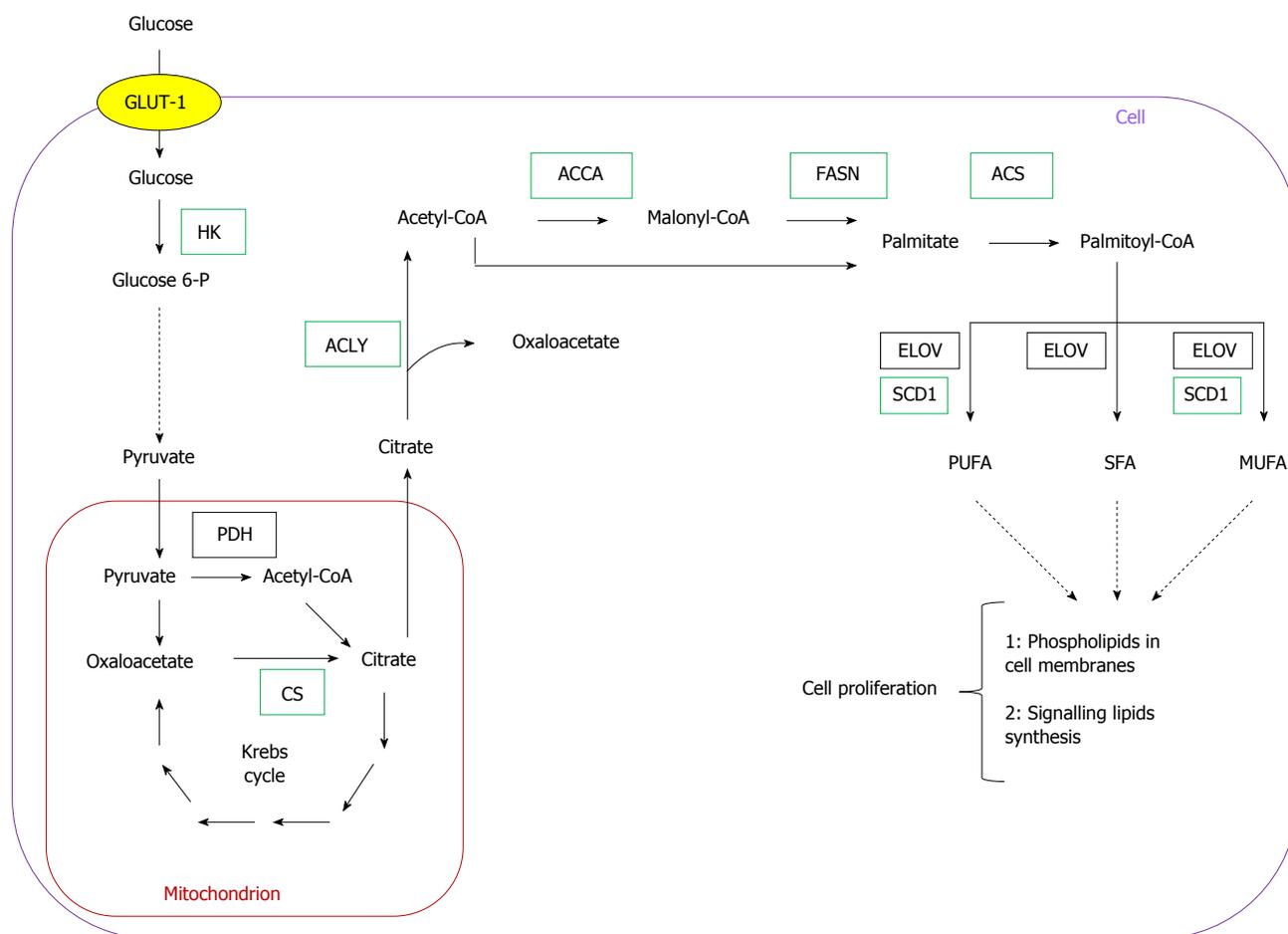
Figure 5 Fatty acid synthesis in pancreatic cancer. The up-regulated enzymes are marked in green. GLUT-1: Glucose transporter 1; HK: Hexokinase; glucose 6-P: Glucose 6-phosphate; PDH: Pyruvate dehydrogenase; CS: Citrate synthase; ACLY: ATP citrate lyase; ACCA: Acetyl-CoA carboxylase; FASN: Fatty acid synthase; ACS: Acyl-CoA synthetase, ELOV: Elongase; SCD: Stearoyl-CoA desaturase; PUFA: Polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids.

enzyme of oxidative phase, controlling NADPH production) is increased putatively, due to p53 deficiency[19]. p53 inhibits G6PDH through direct binding, and its loss leads to the up-regulation of the oxidative PPP phase in cancer cells[73,124]. Taken together, these data suggest that similar to other malignancies, the increased glucose flux (both by glycolytic and pentose phosphate pathway) is integrated with the enhanced biosynthesis of lipids in PC cells. Pathways involved in the conversion of glucose to lipids in PC cells are presented in Figure 5.

The lipids formed in cancer cells play two important roles. Firstly, they are building blocks for cell membrane formation during cell proliferation (mainly cholesterol, phosphatidylcholine, phspatidylserine, phosphatidylethanolamine). Secondly, they play an important role as signaling molecules (phosphatidylinositol, phosphatidic acid, diacylglycerol), or substrates for posttranslational protein modification, including palmitoylation and prenylation[125]. Mammalian cancer cells rely mostly on saturated (SFAs) or monounsaturated FAs (MUFAs). MUFAs are less susceptible to peroxidation, thus increasing the resistance of cancer cells to oxidative stress[126]. Elevated level of MUFAs is maintained mostly by stearoyl-CoA desaturase 1 (SCD1). Inhibition of SCD1 activity in some tumors

(*e.g.*, in prostate cancer) leads to inhibition of cancer cell growth. Diminished SCD1 activity is reflected by lower synthesis of phosphatidylinositol, which participates in AKT activation, crucial for cancer development and growth. Additionally inhibition of SCD1 blocks oncogenic transformation of KRAS necessary for activation of this gene and further tumor growth[127]. As SCD1 is very active in PDAC cells[128], and KRAS and AKT signaling pathway are important for their development and growth, SCD1 supposedly plays an essential role in pathogenesis of that malignancy via the same mechanism as in case of other tumors.

In the context of lipid synthesis, especially FASN activity, special attention should be paid to lipid rafts. Lipid rafts are cholesterol- and sphingolipid-rich membranous lipid domains, which contain several signaling and transport proteins. According to some authors, lipid rafts play an important role in health and disease, including carcinogenesis[129]. Lipid rafts rich in proteins of the caveolin family are referred to as caveolae. Caveolin-1 encoding gene expression is altered in some cancers including colon cancer[130,131], breast cancer[132], urothelial carcinoma[133], esophageal squamous cell carcinoma[134], and prostate cancer[135]. The overexpression of caveolin -1 in
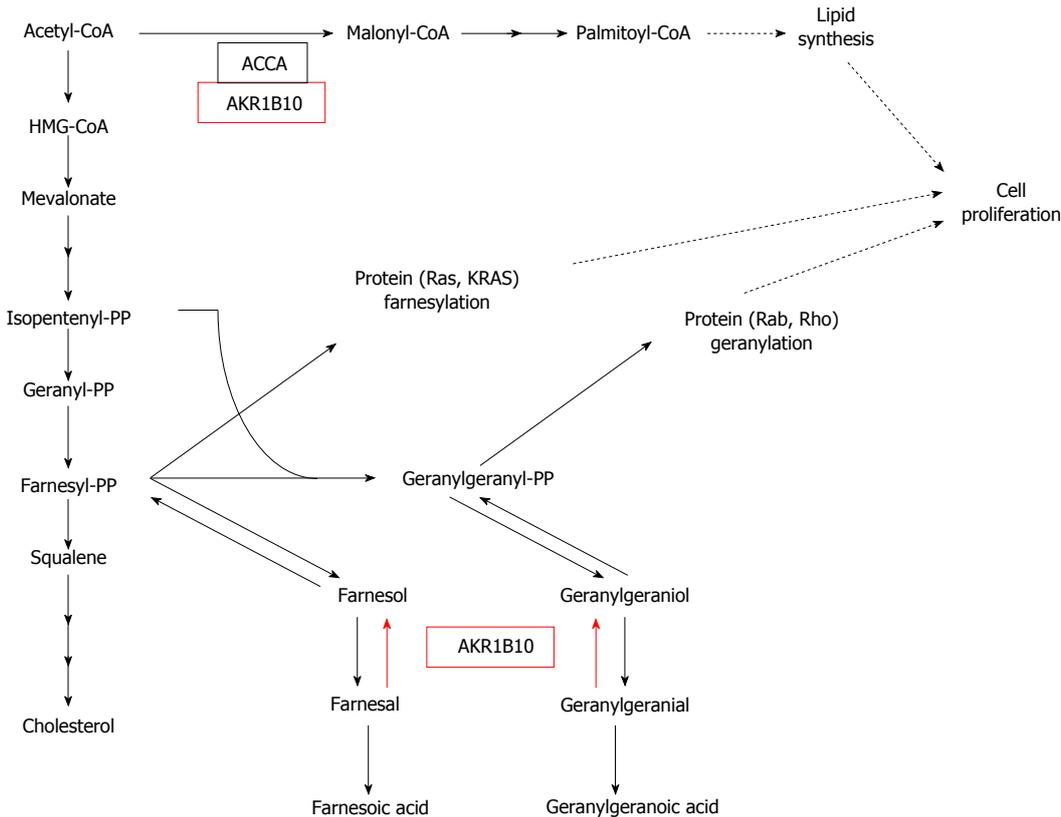
Figure 6 Possible role of aldo-keto reductase 1B10 in regulation of cell proliferation in pancreatic cancer. Binding of AKR1B10 results in stabilization of ACCA, and up-regulation of fatty acid synthesis. AKR1B10: Aldo-keto reductase family 1B10; ACCA: Acetyl-CoA carboxylase; Isopentenyl-PP: Isopentenyl diphosphate; Geranyl-PP: Geranyl diphosphate; Farnesyl-PP: Farnesyl diphosphate; Geranylgeranyl-PP: Geranylgeranyl diphosphate.

colon cancer cells is associated with elevated saturated to unsaturated FA ratio in cellular membrane[136]. Deregulation of caveolin-1 is also observed in PC cells[137]. Moreover, caveolin-1 and *FASN* are co-expressed in the cells of this malignancy. This phenomenon is consistent with histological grade and stage of the tumor (high expression of ceveolin-1 and *FASN* genes correspond with poor differentiation status)[104]. Thus, FASN and caveolin-1 were suggested as potential diagnostic and prognostic markers of PC and possible therapeutic targets[104].

Recently published data suggest that cancer cells do not rely solely on the *de novo* lipogenesis, but also utilize food-derived FAs for synthesis of phospholipids required for cell proliferation and lipid signaling[138,139]. This corroborates well with the evidence that a high dietary intake of fat constitutes potential risk factor of some malignancies[140]. Moreover, there is growing evidence that obesity, associated with elevated blood concentrations of FAs, modulates the risk and prognosis of certain cancers[141]. These findings suggest that, apart from lipogenesis, cancer cells can utilize FAs present in blood (derived from VLDL and chylomicrons or from adipose tissue) for their growth. This fact may partly explain why many promising lipogenic enzymes inhibitors tested succesfully in preclinical studies did not confirm their efficacy in further clinical trials. Furthermore, apart from inhibition of lipogenesis, also reduced dietary lipid digestion and absorption, and decreased lipoprotein lipase and FAs uptake

seem necessary for the control of cancer growth[139].

Recently, overexpression and oncogenic function of aldo-keto reductase family 1B10 (AKR1B10; A-aldo, K-keto, R-reductase), tightly associated with lipid metabolism in human PC cell lines, has been reported[142]. AKR protein family consists of enzymes which catalyze the reaction: alcohol + NADP$^+$ → aldehyde (or ketone) + NADPH + H$^+$. These enzymes are expressed in numerous human organs/tissues. AKR1B10, the enzyme specific to such substrates as farnesal, geranylgeranial, retinal, and carbonyls[143-146], is overexpressed in certain malignancies, especially in tobacco-related cancers, including non-small cell lung carcinoma[147] and PC[142]. Oncogenic function of AKR1B10 is associated with protein farnesylation and up-regulation of FA synthesis by stabilization of ACCA[142,148]. Farnesyl diphosphate is a precursor of cholesterol biosynthesis and a substrate for protein farnesylation, which plays an important role in carcinogenesis[149]. Conversion of farnesyl diphosphate to farnesol diminishes its intracellular level, and, consequently, protein farnesylation. Farnesol can be further converted to farnesal, then oxidized to farnesoic acid. If the activity of AKR1B10 is high (as in the case of PC cells), farnesal is reduced to farnesol, following the reaction pattern: farnesal + NADPH + H$^+$ → farnesol + NADP$^+$. Farnesol can be re-phosphorylated to farnesyl diphosphate, increasing the ability for protein farnesylation (Figure 6). Farnesyl diphosphate, together with isopentenyl di-

phosphate, is converted to geranylgeranyl diphosphate, a substrate for protein geranylation. A geranylgeranyl diphosphate, *e.g.*, farnesyl diphosphate, can be converted to geranylgeranoic acid (*via* geranylgeraniol and geranylgeranial) (Figure 6). Similarly, high activity of AKR1B10 may cause the reversed conversion of geranylgeranial to geranylgeranyl diphospate, a substrate for protein geranylation (Figure 6). siRNA-mediated silencing of AKR1B10, knockdown of AKR1B10, or inhibition of the enzyme activity lead to decrease in protein prenylation[142]. Membrane-bound KRAS protein of PC cells, a product of point mutation in KRAS is activated by prenylation. If the expression of AKR1B10 is diminished, the activity of membrane-bound KRAS protein decreases in pancreatic cell lines[142]. Thus, the deactivation of AKR1B10 and resultant inhibition of the prenylation (fanezylation, geranylgeranylation) of protein (*e.g.*, KRAS), may constitute a promising target for PC treatment.

3-hydroxy-methylglutaryl-CoA reductase (HMG-CoA reductase) is a key enzyme of cholesterol synthesis pathway (Figure 6), which is inhibited by statins, prescribed to treat hypercholesterolemia. Since the reaction catalysed by HMG-CoA reductase provides substrate for cholesterol synthesis (that is of great importance in rapidly proliferating cancer cells), and also for isoprenoids necessary for prenylation of proteins, the application of statins as an antiproliferative drugs have been studied. Numerous *in vitro* studies, also with the use of PC cancer cells, provided promising results[150].

Cyclooxygenase-2 (COX-2) is another enzyme which plays an important role in lipid metabolism, namely in the conversion of arachidonic acid (released from membrane phospholipids by phospholipase A₂) to prostaglandins. COX-2 is overexpressed in many malignancies, including 45%-75% PCs[151-155]. This suggests, that this enzyme plays an important role in pancreatic carcinogenesis and chemoresistance of PC cells. Moreover, the overexpression of COX-2 in PC cells was postulated to be associated with greater invasiveness of this malignancy and promotion of angiogenesis. Recent data suggest that combination of COX-2 inhibitor (Celecoxib) with gemcitabine and irinotecan could be an active treatment for non-operable PC[152]. These clinical observations have been supported by the results of *in vitro* studies. Inhibition of COX-2 by non-steroidal anti-inflammatory drugs causes a dose-dependent block of pancreatic cell line proliferation[156]. According to recent reports, the anti-tumor activity of class I histone deacetylase (HDAC) inhibitors in human PC model is significantly improved by the simultaneous inhibition of COX-2[157]. Taken together, the results of clinical and *in vitro* observations suggest that COX-2 plays an important role in PC development. The up-regulation of COX-2 in PC cells and its role in carcinogenesis are probably related to inflammation. The anti-cancer action of COX-2 inhibitors is most likely associated with the reduction of inflammation that can contribute to cell proliferation. Several authors revealed that many malignancies, including PC, result from a chronic inflammatory process[158]. According to Jackson and Evers[151],

several signaling pathways involving COX-2, NF-kappa B and phosphatidyl inositol 3-kinase may constitute a link between inflammation and carcinogenesis.

ABNORMAL LIPID METABOLISM AND CANCER PROGRESSION AND PROGNOSIS

Overexpression of *FASN* is associated with significantly enhanced proliferation of non-tumorigenic mammary[111] and prostate[159] epithelial cells. On the other hand, siRNA-mediated silencing of *FASN* gene expression or inhibition of FASN activity by pharmacological (synthetic or natural) agents leads to growth arrest of some cancer and normal cells[160-162]. Moreover, FASN inhibitors suppress the synthesis of DNA and induce apoptosis in cancer cell lines[163]. Previous studies confirmed the association between FASN activity and cell cycle progression[161,164]. However, activity of FASN was not reflected by cell cycle progression in some experimental models, *e.g.* MCF7 cell line[165]. Also siRNA-mediated knockdown of *FASN* gene expression did not cause a significant growth arrest in PC cell line (Panc-1)[105]. Therefore, the results published thus far do not present sufficient evidence for the role of FASN in cell cycle regulation, especially in PC cells. Nevertheless, the FASN knockdown in Panc-1 cells were revealed to show reduced resistance to gemcitabine[105].

The results of *in vitro* studies and clinical observations suggest that elevated expression of *FASN* gene in cancer cells is related to markedly worse prognosis. Overexpression of *FASN* gene was proved to be associated with cancer progression, higher risk of recurrence and shorter survival of patients with breast cancer[5,166], prostate cancer[167], melanoma[168], nephroblastoma[169], renal cell carcinoma[170], endometrial carcinoma[171], colorectal carcinoma[172], ovarian cancer[173], squamous cell carcinoma of the lung[174], head and neck squamous cell carcinoma[175], and squamous cell carcinoma of the tongue[176]. CD44 is a transmembrane glycoprotein which is involved in tumor progression and metastasis[177]. Interaction between CD44 and c-MET (tyrosine kinase), a proto-oncogene involved in several processes (including tumor growth, invasion, and metastasis)[178], is essential for activation of the latter and down-stream signaling in some malignancies[179]. Interestingly, inhibition of FASN and ACLY in human colorectal cancer cell lines (KM20, HCT116) is associated with reduced expression of CD44. This is attributed to attenuated activation of c-MET, AKT, FAK, and paxillin, factors affecting adhesion, migration and invasion of cancer cells[180]. The abovementioned phenomenon was reflected by lower metastatic potential of colorectal cancer cells. The data suggest a direct link between lipogenic enzyme activity (FASN and ACLY) and tumor progression to a metastatic phenotype. As the inhibition of FASN is related to decreased phosphorylation of c-Met in diffuse large B-cell lymphoma[181] and prostate cancer[182], one can surmise that lipogenesis is feature of

metastatic cancers, including PC. However, to date there are no evidence confirming this hypothesis.

Overexpression of *FASN* gene is associated with poor prognosis in PC patients[96,104,105]. As previously mentioned, the overexpression of *FASN* gene may be associated with gemcitabine resistance of PC cells[105], and the inhibition of FASN enhances the cytotoxicity of this agent[105]. Similar phenomenon was observed in the case of human breast cancer cells and ovarian cancer cells. According to Menendez group, the inhibition of FASN is associated with enhanced cytotoxicity of docetaxel, vinorelbine, paclitaxel, 5-fluorouracil, and herceptin in the Her-2 positive breast cancer cell lines and ovarian cancer cells[183-187].

SERUM FATTY ACID SYNTHASE LEVEL AND SERUM FATTY ACID PROFILE-POTENTIAL BIOMARKERS FOR PANCREATIC CANCER

At present there is no sufficiently specific and sensitive serum (plasma) marker of PC. Ca19-9, the most widely used marker of this malignancy (the sensitivity up to 80%), is also elevated in other conditions, including chronic pancreatitis and cholangitis, as well as in other tumors[188,189]. Moreover, Ca19-9 is not useful in detecting early stages of PC[190]. According to some authors, circulating micro-RNA (miR-21, mir-210, mir155, mir196a) could constitute novel diagnostic biomarkers of PC[191,192]. Proteomic analyses of human PCs revealed numerous differentially regulated proteins, which could be involved in the progression of this malignancy, and, consequently, could act as its biomarkers, determined in pancreatic juice and in serum[193]. Also up-regulation of numerous proteins, which can be used as biomarkers of PC, has been reported recently[194]. However, despite extensive studies, we still lack a valid approach for detection of PC, especially its early stages, and sufficiently specific and sensitive biomarkers of this malignancy.

The fact that cancer cells and the normal cells of surrounding tissues are characterized by differential expression patterns of *FASN* suggests that serum levels of FASN may constitute a good biomarker of malignancy. Indeed, up-regulation of *FASN* in cancer cells was proved to be associated with increased serum levels of this enzyme in patients with some malignancies. The serum FASN level measured by ELISA in breast, prostate, colon, and ovarian cancer patients was significantly higher than in healthy controls[195-197]. Moreover, an increase in the serum levels of FASN proved to be proportional to the clinical stage of colorectal cancer and breast cancer[196,198]. The ELISA-determined serum levels of FASN were also elevated in patients with PC and intraductal papillary mucinous neoplasm[98]. Interestingly, the serum FASN levels of most PC patients decreased after resection of this malignancy[98]. This suggests that the elevated serum level of FASN reflects its up-regulation in PC

cells. However, increased levels of FASN were also found in sera of patients with chronic pancreatitis[98]. This suggests that this parameter is not a PC-specific biomarker. Nevertheless, the serum levels of FASN could potentially add to the panel of markers used in the monitoring of individuals at high risk of PC.

According to some authors, PC patients show increased proportion of total MUFA in all plasma lipid classes, a feature which is associated with increased delta 9 desaturase (SCD1) and delta 5 desaturase indices[128]. Moreover, the association between longer survival of PC patients and higher level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and with lower SCD1 index was demonstrated[128]. Recently, Yabushita *et al*[199] documented a significant decrease in serum (and pancreatic) level of palmitoleic acid in an experimental model of PDAC, and suggested that this FA could serve as a biomarker of human PC. Palmitoleic acid is a monounsaturated FA (16:1 n-9). It can be synthesized from palmitic acid, the main product of FASN, or may originate from diet. Conversion of palmitic acid to palmitoleic acid is catalyzed by SCD1, which is up-regulated in some malignancies including PC[128,200]. The reason for decrease in palmitoleic acid in patients with PC is not clear, as due to higher activity of SCD1, elevated level of this FA should be rather anticipated. Despite unknown molecular basis for the decreased serum and tissue concentration of palmitoleic acid the diagnostic value of this finding should be verified in patients with PC. Chavarro *et al*[201] showed that blood levels of some MUFAs including myristoleic acid (14:1 n-5), palmitoleic acid, and oleic acid (18:1 n-9), were associated with higher incidence of prostate cancer. This relationship was the strongest in the case of palmitoleic acid.

Recently Zhang *et al*[202] reported that PC can be diagnosed by means of ^{1}H nuclear magnetic resonance (NMR)-based metabonomic profiles. These authors showed that numerous plasma metabolites, including lipids, are either elevated (*e.g.*, VLDL) or decreased (*e.g.*, HDL, LDL and 3-hydroxybutyrate) in patients with this malignancy.

Yabushita *et al*[199] revealed that serum chenodeoxycholic acid, a major constituent of bile acids (which play a key role in lipid digestion in the alimentary tract), is elevated in the experimental model of PDAC. Also Urayama *et al*[203] claimed on elevated serum levels of some bile acids (taurocholic acid and tauroursodeoxycholic acid) in PC patients.

Overall, several PC-characteristic features of lipids metabolism have been found: (1) elevated serum level of FASN; (2) elevated serum levels of EPA, DHA and VLDL; (3) decreased serum levels of palmitoleic acid, HDL, LDL and 3-hydroxybutyrate, and (4) elevated serum levels of bile acids. All these parameters could serve as additional markers of PC.

Up-regulation of lipogenic enzymes in PC cells and resultant enhanced synthesis of lipids[19,96,104,105] seem to occur early in tumorigenesis and can be associated with

Table 5 Lipogenic enzyme inhibitors that can be used as potential antitumor drugs

Enzyme name	Inhibitor	Type of neoplasm	Ref.
Fatty acid synthase	Cerulenin	Breast cancer,	[303]
(FASN)		ovarian cancer	[304]
	C75	Breast cancer	[216]
		Pancreatic cancer	[232]
	Epigallocatechin-3-gallate (EGG)	Prostate cancer	[228]
	C93	Lung cancer	[305]
		Ovarian cancer	[306]
	Luteolin	Breast cancer, ovarian cancer	[228]
		Pancreatic cancer	[232]
	Orlistat	Prostate cancer	[307]
ATP citrate lyase	SB-204990	Lung cancer	[308]
(ACLY)	hydroxycitrate	Brest cancer	[18]
		Pancreatic cancer	[101]
Acetyl-CoA carboxylase	Soraphen A	Prostate cancer	[309]
(ACCA)	TOFA	Lung cancer, colon cancer	[310]
Stearoyl-CoA desaturase	CVT-11127	Lung cancer	[222]
(SCD1)	TOFA	Colon cancer	[225]
Acetyl-CoA synthetase (ACS)	Triacsin c	Various cancers cell lines	[311]

the progression of the disease. Therefore, metabolic imaging with lipid precursor tracers: ^{11}C-acetate, ^{18}F-fluoroacetate (as a substrates for FA synthesis), and ^{11}C-choline, ^{18}F-fluorocholine (as a substrate for phosphatidylcholine synthesis), may constitute a novel imaging technique for diagnosis of PC, even at the very early stages of this malignancy. It is of note that ^{11}C-acetate and ^{11}C-choline have been successfully used for detecting primary prostate cancer, as well as metastases and recurrence of this malignancy[204]. However, both ^{11}C-acetate and ^{11}C-choline cannot be used in case of small metastatic foci[205]. Moreover, the sensitivity of ^{11}C-acetate in the detection of prostate cancer is decreased in patients whose PSA level is lower than 3 ng/mL[204]. Finally it should be remembered that the incorporation of ^{11}C-acetate (or its analogue ^{18}F-fluoroacetate) to lipids is determined not only by FASN activity, but also by the activity of acetyl-CoA synthetase[206].

ABNORMAL LIPID METABOLISM AS A PROMISING TARGET OF PANCREATIC CANCER TREATMENT

Chemotherapy provides only modest improvement in pancreatic cancer patients. Effective molecular therapeutic strategy requires characteristic features of the disease to be identified. As previously mentioned the values of some parameters of lipids synthesis, namely the expression of *FASN* gene and resultant activity of FASN, are significantly higher in cancer cells than in adjacent normal cells. This suggests that inhibition of FASN could constitute a selective therapeutic approach in cancer patients. Possible application of FASN as a therapeutic target is sustained by the results of many studies which showed that pharmacological blockade of this enzyme exerted cytostatic and cytotoxic effects to several tumor cells[97,109,125,207-216]. Pharmacological blockade of

other enzymes involved in lipogenic pathway such as ACLY[18,209,217,218], ACCA[219-221], SCD1[222-225], and acyl-CoA synthetase[209], could also be an effective strategy for cancer treatment. Table 5 lists lipogenic enzymes inhibitor which can be potential antitumor drugs.

Similar to other malignancies, the overexpression of *FASN* observed in PC cells is associated with poor prognosis[96,104]. This suggest that FASN is involved in PC cell survival and its inhibition could constitute an effective strategy for PC treatment. Irresponsiveness to chemotherapy and radiotherapy is an important feature of PC. According to Yang *et al*[105], overexpression of *FASN* can be associated with resistance to gemcitabine and radiotherapy in PC patients. The exact molecular mechanism by which FASN induce gemcitabine resistance of PC cells is unknown. As the elevated expression of this molecule was proved to protect breast cancer cells from drug-induced apoptosis[165], also the FASN-induced resistance of PC cells to gemcitabine can result from similar mechanism. C75 (trans-4-carboxy-5-octyl-3-methylenebutyrolactone), a synthetic analog of natural cerulenin (isolated from Cephalosporum caerulens), is an inhibitor of FASN most often used in experimental models. This antitumor activity of this agent was documented in the case of human breast cancer[109], prostate cancer[226], ovary cancer[227] and mesothelioma[215] cell lines. Also many green tea polyphenols (*e.g.*, EGCG-epigallocatechin gallate or ECG-epicatechin gallate) and plant-derived flavonoids (such as luteoin) showed inhibitory effect to FASN[208]. Green tea polyphenols down-regulate *FASN* gene expression and induce apoptosis in human prostate cancer[228-230]. Luteolin (natural flavonoid) inhibits FASN *in vitro* and induces cytotoxic effects in breast, prostate cancer and hepatocellular carcinoma cells[231]. Moreover, the consumption of flavonoid rich foods was revealed to decrease the incidence of some malignancies[105]. Harris *et al*[232] studied the effect of FASN inhibitors (C75 and some phytochemicals) on the *in vitro* proliferation of PC

cells (MIA PaCa-2). They found that C75 and luteolin decreased proliferation of these cells at a similar dose. Also other tested phytochemicals, quercetin (flavonoid) and resveratrol (stilbenoid), inhibited the proliferation albeit, at significantly higher concentrations. The same authors revealed that the inhibitory effect of luteolin against PC cells results from three mechanisms: decreased synthesis of FA, and nucleic acids and decreased energy production. In contrast quercetin and resveratrol (natural inhibitors of FASN), which showed weaker inhibitory potential affect mainly glycogen metabolism. Collectively, the results published by Harris *et al*[232] suggest that the blockade of FASN by some flavonoids could lead to inhibition of pancreatic cells proliferation, similarly as in other cancer cells.

The results of clinical observations suggests that the incidence of cancer in diabetic patients, treated with metformin (an oral hypoglycemic drug, N,N'-dimethyl biguanide) is lower than in individuals with diabetes who do not receive this drug[233-236]. The anticancer properties of metformin were also confirmed by *in vitro* studies[237,238]. Recently, Nair *et al*[239] reported that metformin inhibits PC cell proliferation and tumor growth via down-regulation of Sp transcription factors and Sp regulated genes. Noticeably, *FASN* is one of the Sp regulated genes[240]. Thus, one can assume that the metformin induced blockade of PC cell proliferation and tumor growth is at least partially associated with indirect inhibition of FASN activity and lipid synthesis.

The anticancer potential of statins, inhibitors of HMG-CoA reductase also have been studied *in vitro* with various cancers cells lines. The antitumor effects of lipophilic statins (*e.g.*, lovastatin, simvastatin) resulted mainly from suppression of proliferation and promotion of apoptosis[150]. The chemopreventive effects of statins have been also reported in PC cell lines[241-243], and in mouse model of PC[244]. Available data from analyses on large human populations show, that daily intake of statins, in doses for cardiovascular event prevention, is not associated with the risk of PC[245-247]. However some recent data suggests that in subgroup of male smokers statins use may reduce the odds of PC[248], and is associated with better survival in diabetic patients[249]. The combination of statins and a FASN inhibitors used in an anticancer therapy would be of particular interest, but until now there are no data published regarding such approach.

In summary, the results presented above suggest that inhibitors of FASN (and inhibitors of other lipogenic enzymes) constitute promising anticancer agents. However, most of the known FASN inhibitors which can be potentially used as anticancer drugs displayed some side effects[250]. Nevertheless, the evidence of PC cells proliferation blockade resulting from direct or indirect inhibition of FASN, and potential involvement of FASN in gemcitabine (chemotherapeutic) resistance, substantiate further research on the role of this molecule in the biology and therapy of pancreatic malignancies. Moreover, there is an urgent need for specific/selective, side effect free inhibitors of FASN, which can be used in treatment of PC.

CONCLUSION

Similar to other malignancies, the reprogramming of lipid metabolism in PDAC, is closely connected with tumor development, growth, and progression. Hypoxia, activity of oncogenic factors, or the loss of tumor suppressors lead to significant changes in lipid biosynthesis and metabolism. KRAS, together with MYC and HIF1α, either increase the use of glucose and glutamine as substrates for FA synthesis, or regulate the lipogenesis directly. SFA and MUFA, (produced by FASN and SCD1 or taken up from blood), enhance the tumor growth by up-regulation of some oncogenic factors. FA built into phospholipids (together with caveolin-1) participate in the remodeling of cancer cell membrane structure. Other products of altered lipid metabolism, such as isoprene derivatives (farnesyl diphosphate or geranylgeranyl diphosphate), influence the activity of some proteins involved in tumorigenesis (enzymes and regulatory proteins) through their prenylation. Up-regulation of prostaglandin biosynthesis (from arachidonic acids) by COX2 links inflammation to PC development.

FASN is the most extensively studied enzyme involved in the lipid metabolism of PDAC cells. Its high activity in PC cells is associated with poor prognosis and increased resistance to chemo- or radiotherapy. Elevated serum levels of FASN, EPA, DHA or VLDL, and decreased serum levels of palmitoleic acid, HDL, LDL, or 3-hydroxybutyrate could serve as additional markers of PDAC. As the lipogenic activity of PDAC cells is higher than in normal cells, pharmacological inhibition of FASN and other lipogenic enzymes seems a promising therapeutic target. C75, some flavonoids, and metformin are good candidates for anticancer agents, but further research is required prior to their implementation to PDAC treatment.

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Anaesthetic perioperative management of patients with pancreatic cancer

Lesley De Pietri, Roberto Montalti, Bruno Begliomini

Lesley De Pietri, Bruno Begliomini, Division of Anaesthesiology and Intensive Care Unit, Azienda Ospedaliero-Universitaria di Modena-Policlinico, 41100 Modena, Italy

Roberto Montalti, Transplantation Surgery, Azienda Ospedaliero-Universitaria Ospedali Riuniti di Ancona, 60126 Modena, Italy

Author contributions: De Pietri L conceived and designed the review; Montalti R drafted the article; Begliomini B revised the manuscript.

Correspondence to: Dr, Lesley De Pietri, Division of Anaesthesiology and Intensive Care Unit, Azienda Ospedaliero-Universitaria di Modena-Policlinico, No. 71 via del Pozzo, 41100 Modena, Italy. lesley.depietri@yahoo.it

Telephone: +39-59-4225864 Fax: +39-59-4223765

Abstract

Pancreatic cancer remains a significant and unresolved therapeutic challenge. Currently, the only curative treatment for pancreatic cancer is surgical resection. Pancreatic surgery represents a technically demanding major abdominal procedure that can occasionally lead to a number of pathophysiological alterations resulting in increased morbidity and mortality. Systemic, rather than surgical complications, cause the majority of deaths. Because patients are increasingly referred to surgery with at advanced ages and because pancreatic surgery is extremely complex, anaesthesiologists and surgeons play a crucial role in preoperative evaluations and diagnoses for surgical intervention. The anaesthetist plays a key role in perioperative management and can significantly influence patient outcome. To optimise overall care, patients should be appropriately referred to tertiary centres, where multidisciplinary teams (surgical, medical, radiation oncologists, gastroenterologists, interventional radiologists and anaesthetists) work together and where close cooperation between surgeons and anaesthesiologists promotes the safe performance of major gastrointestinal surgeries with acceptable morbidity and mortality rates. In this review, we sought to provide simple daily recommendations to the clinicians who manage pancreatic surgery patients to make their work easier and suggest a joint approach between surgeons and anaesthesiologists in daily decision making.

Core tip: Currently, the only curative treatment for pancreatic cancer is surgical resection. However, this type of surgery is still burdened by considerable morbidity due to its complexity and to the type of referred patients (elderly and with many co-morbidities). We believe that anaesthetic management with proper surgical approaches can play a key role in the outcome of the patient. Simple perioperative precautions in anaesthetic management (patient risk assessment, fluids management, prevention of surgical site infection, thromboprophylaxis, intraoperative ventilation, and intensive postoperative management) can help to ensure that these surgical operations are performed with reasonable assurance.

INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer-related death in the United States and the sixth in

Table 1 A schematic representation of the integrated management of perioperative patients undergoing surgery for pancreatic cancer		
Preoperative	**Intraoperative**	**Postoperative**
Informed patient consent	Combined general and epidural analgesia	Early nasogastric tube, catheter and drain removal
Preoperative risk assessment	Prevention of surgical site infection: Antimicrobial prophylaxis	Early oral nutrition/glycaemic control/goal-directed fluid therapy
	Avoid hypothermia Glucose control	Pain relief/non-opioid oral analgesia
Evaluation and optimisation of preoperative physical conditions and medications	Blood transfusion management	Intensive postoperative ambulation and prevention of venous thromboembolism
Nutritional status	Intraoperative fluid management	Intensive respiratory rehabilitation
Risk stratification, rationale for thromboprophylaxis, and recommendations	Optimisation of intraoperative ventilation Intraoperative thromboprophylaxis	Intensive postoperative management

Modified from Grade *et al*[10].

Europe, with the lowest survival rate for any solid cancer worldwide[1]. It is the most lethal type of digestive cancer and exhibits a five year survival rate of 5% with a range that is correlated with staging and location. The main reason for this extremely poor prognosis is that less than 15% of patients are diagnosed with resectable tumours[2]. Currently, the only curative treatment for PC is surgical resection, although even for resectable tumours, cure is still rare (5-year survival rate of approximately 15%-20%)[3].

Pancreatic surgery represents a challenging and technically demanding major abdominal procedure that occasionally results in a number of pathophysiological alterations during the early postoperative period that account for increased rates of morbidity and mortality.

Systemic, rather than surgical complications, cause the majority of PC-related deaths[4]. More than 80% of PCs are diagnosed in patients older than 65 years. Many PC patients are or have been heavy smokers[5,6], and nearly 80% of PC patients have either frank diabetes or impaired glucose tolerance[7]; venous thromboembolism remains a major complication of PC[8]. For these reasons, PC patients who undergo a major abdominal surgery are at increased anaesthesiological risk. In the light of these issues, it is important to refer these patients to centres with a high volume of operations where a multidisciplinary approach is applied to improve the overall outcome. Moreover, careful patient selection is fundamental.

In this setting, the anaesthesiologist plays a crucial role during preoperative evaluation, which together with a proper surgical approach and a concerted effort with medical physicians, radiation oncologists, gastroenterologists and interventional radiologists is crucial for a favourable perioperative outcome[9]. Patient outcome can be significantly influenced by anaesthesiological management (Table 1), starting with patient stratification and selection, continuing throughout the surgical operation and finishing with postoperative care [intensive care unit (ICU), recommendations for the ward][10].

PREOPERATIVE MANAGEMENT

Informed patient consent
Despite recent developments in operative technique and postoperative care, pancreatic surgery remains associated with high morbidity and mortality. Postoperative complications such as primarily pancreatic fistula, haemorrhage, abscess, and delayed gastric emptying still occur at a frequency of 30% to 60%, resulting in a mortality rate of 1% to 5%[11]. For this reason and due to the lethality of the pancreatic cancer despite surgical treatment, the patient should be informed about the therapeutic procedure and any potential complications or disabilities to facilitate a conscious involvement in the decision-making process.

In the case of patients of advanced age who require pancreatic surgery, formal mental status testing can help determine whether a patient can be considered capable of making this type of decision.

Dementia is an extreme predictor of poor outcome, exhibiting surgical mortality rates that are increased by 52%[12]. The decision to classify an elderly patient eligible for surgery cannot exclude preoperative mental status.

Preoperative risk assessment
A complete history, physical, laboratory examinations, and an assessment of the surgical risks should be included in the preoperative evaluation of an elective surgery.

Currently, the definition of preoperative risk remains vague and difficult to standardise, as it is influenced by many variables attributed to patient- and surgery-specific variability[13]. Recently, a variety of scoring systems has been developed, and the Physiologic and Operative Severity Score for the Enumeration of Mortality and morbidity (POSSUM) model by Copeland *et al*[14] was recognised as the most effective for general surgery[15]. This model, which uses scores relating to 12 physiological and 6 operative variables, was developed to postoperatively predict 30-d mortality and morbidity. The application of the predictive POSSUM and P-POSSUM (Portsmouth modification of POSSUM)[16] models to cases of pancreatic surgery has generated conflicting results. The implementation of this scoring system in the routine practice has proven to be difficult, and a recent review by Wang *et al*[17] has found POSSUM to overpredict postoperative mortality. Despite these limitations, there is still a role for POSSUM as a useful tool in pancreatic surgery. Individual POSSUM scores should not preclude pancreatic resection in clinical practice but might help surgeons modify

expectations of postoperative outcomes[18].

Due to the limitations of the POSSUM model, more trials are needed to adequately evaluate this scoring system in predicting postoperative mortality for pancreatic surgery.

Evaluation and optimisation of preoperative physical conditions and medications

A growing number of old patients benefits from a surgical procedure[19]. Age is an independent risk factor of postoperative mortality and postoperative complications and can cause a gradual progressive loss in the biological reserves for maintaining physiological homeostasis under stress. In addition, an increasing number of patients present with one or more age-related chronic conditions, which further decrease their ability to respond to stress. Cardiac and pulmonary diseases are the most frequently observed co-morbidities that anaesthetists and surgeons must manage during this complex surgery.

A complete history of prior medical and surgical conditions and a full medication list are particularly important[20,21].

Cardiovascular risk evaluation: Cardiovascular complications are among the most common and significant postoperative problems in elderly patients. A practical guideline for perioperative cardiovascular evaluation for non-cardiac surgery has been proposed by the American College of Cardiology and American Heart Association Task Force[22]. Patients should be assessed using an approach that considers clinical predictors, the risk of the proposed operation and the functional capacity.

Ageing is accompanied by increased vascular and ventricular stiffness, diastolic dysfunction and an increased risk of heart failure[23]. Diastolic dysfunction even with a normal or supranormal ejection fraction might elicit a significant effect on the perioperative outcome and management of elderly patients[12]. Diastolic dysfunction might significantly affect perioperative haemodynamics, response to fluid shifts, anaesthetic drugs and other perioperative medications.

Patients with cardiovascular diseases are sensitive to haemodynamic instability and often require increased filling pressures to generate an adequate cardiac output. The anaesthetist must carefully manage fluids during the operation to avoid overload or rapid volume administration. Moreover, the anaesthetist must maintain a normal haemoglobin value (Nair *et al*[24] demonstrated that anaemia was strongly associated with diastolic dysfunction in patients with coronary artery disease) and, if possible, must choose volatile anaesthetics that appear to improve diastolic parameters (in contrast to propofol, which elicits the opposite effect) as measured by echocardiography[25]. Thoracic epidural analgesia should be strongly suggested, not only for pain management and for decreasing respiratory complications but also because its use appears to improve cardiac function by improving the diastolic characteristics of the left ventricle[26,27].

Prophylactic perioperative β-blockade: In general, cardiovascular medication should not be discontinued prior to surgery. In the perioperative setting, β-blockers are not contraindicated in patients with diastolic heart failure and should be continued in patients with systolic heart failure. However, caution is warranted with the acute administration of β-blockers in situations of decompensating systolic heart failure. Nonetheless, given the risk of acute withdrawal, β-blockade in patients with coronary artery diseases or coronary artery disease risk factors should not be discontinued preoperatively. Rather, perioperatively increasing the dosage of the patient's β-blockade regimen would most likely be beneficial[28-30].

If a patient who is scheduled for elective pancreatic surgery requires a new prescription, it should be started at least 1 mo prior to the procedure to allow for dose adjustment[31,32].

Pulmonary risk evaluation: Pulmonary complications such as pneumonia, failure to wean, and postextubation respiratory failure represent the second most frequent types of postoperative complication following wound infection, with an estimated incidence rate ranging from 2.0% to 5.6% following surgery[33,34]. Pulmonary disease increases the risk of postoperative complications, accounting for 40% of postoperative complications and 20% of deaths[35]. Age-related changes, such as increased closing volumes and decreased expiratory flow rates can predispose older patients to pulmonary complications.

Some postoperative pulmonary complication (PPC) predictors after pancreatic surgery are summarised in Table 2 (modified from Canet *et al*[36]).

Identifying the patients who are at high risk for PPCs, can help the anaesthetist to design individually tailored management approaches[37-39]. Pharmacologic measures for managing these complications are either unavailable or limited, and as a result, treatments must be based on physical therapy and respiratory support ventilation.

Finally, the ability to predict PPCs would enable clinicians to give patients more precise risk assessments, thereby facilitating their decision making.

Nutritional status and mechanical bowel preparation

The prevalence of malnutrition is high in patients who are submitted for surgery and ranges from 35% to almost 60%[40]. Malnutrition has been consistently associated with impaired immunity[41] and can lead to increased complications, such as pressure ulcers, delayed wound healing, increased risk of infections, impaired muscular and respiratory functions[42], as well as increased mortality and poor clinical outcomes.

Nutritional status should be determined because nutritional deficiencies are common in patients who have undergone pancreatic resection for malignant tumours. Because malnutrition is potentially reversible with appropriate nutritional support, the early identification of high-risk patients is crucial, and preoperative malnutrition screening is required to identify and to treat the malnutri-

Table 2 Perioperative clinical predictors of postoperative pulmonary complication in pancreatic oncological surgery		
Patient-related factors	**Surgery-related factors**	**Preoperative testing-related factors**
Congestive heart failure	Abdominal surgery	Serum albumin concentration < 2.5 g/dL
ASA score > 2	Surgery duration > 3 h	Anaemia (Hb < 10 g/dL)
Age > 65 yr	General anaesthesia	Low SpO2
Chronic obstructive pulmonary disease	Transfusions	Chest X ray
Functional dependence	Prolonged hospitalisation	
Weight loss		
Impaired sensorium		
Cigarette smoking		
Respiratory infections within the past month		

Modified from Canet *et al*[36]. ASA: American Society of Anesthesiologists.

tion[43]. Recently, the routine screening of patients to identify risk of malnutrition has been recommended by many national, international, and specialist organisations[44,45]. The malnutrition universal screening tool (MUST) for adults was recently validated by several studies, which have demonstrated that as a screening procedure, MUST is rapid and easy to use[46,47].

The MUST appears to be a valid and easy screening tool for pancreatic surgery[20], which can identify patients at high risk for major complications and death. Furthermore, the MUST can prompt the implementation of effective nutritional interventions to reduce poor outcomes and thereby optimise the use of postoperative critical care beds and hospital resources.

As soon as malnutrition is recognised, preoperative nutritional supplements should be provided when possible. This supplementation can include high-energy foods, vitamins, enteral feedings, or, if necessary, total parenteral nutrition.

Mechanical bowel preparation
"Enhanced recovery" or "fast-track" (FT) programmes, which were first developed by Kehlet[48], are structured interdisciplinary strategies that have been introduced to optimise peri-operative care and to accelerate post-operative recovery[49]. A major intervention principle of this approach is the avoidance of preoperative mechanical bowel preparation (MBP), which has been employed as a preventative measure in gastrointestinal surgery for more than a century as an essential factor for avoiding infectious complications and anastomotic dehiscence. FT programmes, which exclude MBP, have been proposed more often in other surgical fields (elective colorectal, gastro-oesophageal and aortic surgery) and rarely have been applied to liver and pancreatic surgery[50]. The application of MBP in this type of surgery has been evaluated by limited studies (a retrospective case-control study by the Jefferson University[51] and a review by Salvia *et al*[52]), which have shown that it did not improve perioperative

outcomes. At our institution, MBP has been excluded from clinical practice in pancreatic surgery. A recent review examined and compared the application of FT protocols with standard care in elective liver and pancreatic surgeries, showing that FT programmes can enhance post-operative recovery and reduce the length of hospital stays with no increase in adverse events, such as re-admissions, morbidity or mortality[53,54]. The avoidance of MBP, together with other measures including the application of epidural analgesia, the prevention of intra-operative hypothermia, fluid restriction, post-operative nutritional care and early mobilisation, collectively represent essential elements of a FT programme that is warranted for complex surgical operations such as pancreatic resection[55,56]. In our experience FT programmes for hepatopancreatic resections appear to be safe and associated with a reduction in the length of hospital stays.

Risk stratification, rationale for thromboprophylaxis, and recommendations
In patients undergoing general and abdominal-pelvic surgery, the risk of venous thromboembolism (VTE) varies depending on both patient- and procedure-specific factors[57]. Pancreatic cancer is among the most common malignancies associated with thrombosis, as it occurs in 50% of total patients[58]. Prophylaxis against postoperative venous thromboembolism should be tailored to the patient's level of risk. A model (the Caprini score) that can potentially be used for such purposes estimates VTE risk by adding points for various VTE risk factors[59].

Pharmacological prophylaxis reduces the risk of pulmonary embolism by 75% in general surgical patients and by 57% in medical patients[60]. The use of low-molecular-weight heparins (LMWHs) to prevent thrombotic events in these patients is a common and well-documented practice.

Current recommendations strongly advise effective and preventive strategies for all hospitalised patients who are defined as moderate to high risk for VTE and are awaiting pancreatic surgery.

LMWHs appear to be effective and are potentially associated with a lower risk of bleeding when the first dose is administered 12 h preoperatively[57,61]. We recommend the administration of LMWH from the day prior to surgery to all patients scheduled for pancreatic cancer surgery.

In the case of patients who are receiving anticoagulants or antiplatelet therapy and require an elective surgery or procedure, the actual guidelines addressing their management are underlined in Table 3 and are modified from Douketis *et al*[62].

INTRAOPERATIVE MANAGEMENT
Combined general and epidural anaesthesia
The use of thoracic epidurals is widespread for intraoperative and postoperative analgesia. Thoracic epidural anaesthesia (TEA) reduces sympathetic activity, thereby influencing the perioperative function of vital organ systems. Thoracic epidural anaesthesia has been used widely

Table 3 Guidelines on the prophylaxis of venous thromboembolism and antiplatelet and anticoagulant management adjusted according to recent guidelines

In patients receiving bridging anticoagulation with a therapeutic-dose IV of unfractionated heparin, treatment is recommended to be stopped no later than at 4 to 6 h prior to surgery

In patients receiving bridging anticoagulation with a therapeutic-dose of LMWH, the last preoperative dose of LMWH is recommended to be administered at approximately 24 h prior to surgery instead of at 12 h prior to surgery

In patients receiving bridging anticoagulation with a therapeutic-dose of LMWH and are undergoing high-bleeding-risk surgery, resumption of the therapeutic dose of LMWH is recommended at 48 to 72 h after surgery instead of within 24 h following surgery

In moderate-to-high-risk patients receiving acetylsalicylic acid who require non-cardiac surgery, treatment with acetylsalicylic acid is recommended to be continued around the time of surgery instead of discontinued at 7 to 10 d prior to surgery

In patients with a coronary stent who require surgery, deferment of surgery is recommended at 6 wk or 6 mo after the placement of a bare-metal or drug-eluting stent, respectively, instead of initiating surgery during these time periods

In patients requiring surgery within 6 wk or 6 mo of the placement of a bare-metal or drug-eluting stent, respectively, continuing perioperative antiplatelet therapy is recommended instead of stopping therapy at 7 to 10 d prior to surgery

Modified from Douketis *et al*[62]. LMWHs: Low-molecular-weight heparins.

to provide excellent pain relief, to attenuate the catabolic response to abdominal surgery, to lower the incidence of pulmonary morbidity, to decrease the cardiac metabolic demand, to reduce the risk of thromboembolic complications, to promote the recovery of intestinal function and to minimise motor blockade[63,64]. Moreover, epidural anaesthesia and mild hypercapnia have been shown to increase subcutaneous tissue oxygenation[65].

The combination of general anaesthesia and thoracic epidural anaesthesia has become the technique of choice at many institutions for major abdominal surgery[66,67].

Recent studies have suggested that for some types of cancer, TEA might also reduce the rate of recurrence after surgical resection. The possibility of reducing tumour recurrence makes the combination of general anaesthesia and TEA even more appealing, despite the existence of certain contraindications[68,69].

TEA represents a powerful tool that is available to anaesthesiologists for perioperative intervention in pancreatic surgery. At our University Medical Centre, we strongly address its use in the context of multimodal intervention.

Prevention of surgical site infection

Surgical site infections continue to represent a substantial source of morbidity and mortality in the surgical patient population. They are the second most common cause of nosocomial infection after urinary tract infections and account for approximately 17% of all hospital-acquired infections[70].

Increasing evidence indicates that anaesthesiologists play a prominent role in the prevention of surgical site infections. Anaesthesiologists are involved in the administration of antibiotics, in the use of supplemental oxygen, in the maintenance of normothermia and normoglycaemia, in the perioperative fluid management and in the administration of blood transfusions[71,72]. Therefore, decreasing surgical site infections depends on the optimisation of some perioperative conditions, which are generally controlled by anaesthesiologists.

Antimicrobial prophylaxis

The anaesthesiologist can play a simple but effective

role in the prevention of surgical site infections by ensuring the administration of appropriate antimicrobial prophylaxis[73,74].

Current recommendations state that the infusion of the first dose of drug should begin within 30-60 min of incision. This period can be lengthened to 120 min for drugs such as vancomycin, where high infusion rates have been associated with complications[75]. The drugs used should be defined in advance for each intervention, including alternatives in the event that the patient presents with any contraindication for the frontline antibiotics. The determination of the ideal preoperative antibiotic therapy for a patient who is awaiting pancreatic surgery requires efforts by a multidisciplinary team (anaesthesiologist, surgeon and microbiologist). A proper and effective antimicrobial prophylaxis should be based upon the application of a standard protocol and quality management[76].

Concerning the duration and dosage of prophylaxis, the guidelines generally recommended a single standard intravenous therapeutic dose of antibiotic in the majority of procedures. Repeated doses have only been indicated in special circumstances such as prolonged surgery with a duration longer than the half-life of the antibiotic used or cases of major blood loss. This recommendation is based on published evidence, which suggested that the administration of short-duration prophylaxis is equally effective as longer-duration prophylaxis in the prevention of surgical site infections[77,78]. It is advisable to administer at least two antibiotic doses during pancreatic surgery.

Avoid hypothermia

Mild perioperative hypothermia (core body temperature 34-36 °C) is commonly observed in surgical patients. The complications of mild perioperative hypothermia have been studied extensively and include increased duration of hospitalisation, increased intraoperative blood loss and transfusion requirements, increased adverse cardiac events, and an increase in patient thermal discomfort in the recovery room[79,80]. The effects of mild hypothermia on surgical site infections have also been studied. The major relation between hypothermia and increased surgi-

cal site infections is thought to be a decrease in subcutaneous tissue perfusion mediated by vasoconstriction[81,82]. The reduced oxygenation of the wound is responsible for reduced oxidative killing elicited by neutrophils and for the reduced production of superoxide radicals for any given oxygen tension[80].

Intraoperative core temperature monitoring (oesophageal temperature probe) and adequate control of body temperature are essential during pancreatic cancer surgery[83]. Heat loss during the first hour of anaesthesia is generally a result of the redistribution of core-to-peripheral temperature gradients caused by an anaesthetic-induced decrease in vasoconstriction. The exposure of the large bowel, significant amounts of fluids administered, and long surgical procedures represent other causes of intraoperative hypothermia. Actively pre-warming patients for 2 h prior to the induction of either general or regional anaesthesia[80] using forced-air warming blankets together with fluid-warming systems represents an important way to keep patients normothermic[84].

Glucose control

Hyperglycaemia is associated with an increased risk of morbidity and mortality[85]. Several studies have shown the negative effects of hyperglycaemic phases during hospitalisation on the rate of nosocomial infections, length of hospital stay and mortality[71,86]. In a recent trial, the use of insulin infusions to maintain serum glucose at less than 110 mg/dL in critically ill patients decreased the mortality rate from 8.0% to 4.6%, regardless of diabetic status[87]. In subsequent studies, the concept of intensive glucose control was modified towards less-extreme blood glucose levels because of dangerous hypoglycaemic episodes that were attributable for worse patients outcomes than that originally reported[88,89]. Intraoperative glucose control should be a standard practice during long and complex surgical procedures to reduce perioperative complications.

The optimal glucose level during the perioperative period has not been prospectively investigated, and the available data from recent reports do not indicate a specific threshold for the treatment of hyperglycaemia. There is some evidence that keeping glucose levels within a range of 110-180 mg/dL and not limiting the treatment to values higher than 200 mg/dL is safe and appropriate.

It is important not only to limit glucose control during the intraoperative period but also to continue insulin infusion during the postoperative period. The frequent and precise measurement of glycaemia must become a standard of pancreatic cancer patient management both during surgical procedures as well as during the postoperative period[90].

Blood transfusion management

Several published studies have demonstrated how blood product transfusions increase the postoperative risk of infection[91,92].

Published guidelines generally concur that although transfusions are not beneficial when the haemoglobin concentrations are greater than 100 g/L, they confer benefit when the haemoglobin concentrations are less than 60-70 g/L. Studies that have described transfusion management in Jehovah's witnesses have shown that morbidity and mortality only increase postoperatively for each gram of decrement when the haemoglobin concentration is less than 70 g/L[93]. Patients with cardiovascular diseases exhibit a significantly increased rate of postoperative mortality, and for this reason, the transfusion trigger should be different for patients with or without cardiovascular disease[94,95]. Although multiple trials have assessed the effects of transfusion thresholds on patient outcome, the literature is insufficient for defining a transfusion trigger in surgical patients with substantial blood loss. In the light of recent findings, the transfusion management of surgical patients should be patient specific and should not be based on arbitrary laboratory values but guided by patient covariables[96-99]. As underlined by the recent guidelines on perioperative bleeding management of the European Society of Anaesthesiology, we suggest a target haemoglobin concentration of 7-9 g/dL and the guidance of transfusions based on levels of serum lactate, base deficit, and central venous oxygen saturation[100].

Intraoperative fluid management

Optimal perioperative fluid management remains highly challenging, particularly in patients undergoing major abdominal surgery[101-103]. Perioperative physicians generally administer intravenous fluids to replace fasting deficits, third space losses, and blood loss to maintain adequate cardiac output, blood pressure, and urine output.

Fluid excess can have a negative impact on cardiac, pulmonary, bowel function and wound healing, predisposing the patient to tissue oedema and anastomotic breakdown[104,105]. In contrast, excessive fluid restriction can expose the patient to hypovolaemia and hypoperfusion[106]. Surgery causes inflammation and a corresponding release of mediators that can induce local tissue oedema[107]. Anaesthetists generally manage perioperative fluid administration by using unmonitored fixed fluid regimens and estimating fluid loss.

In recent years, restrictive fluid management has replaced this approach, and the concept of fast-track surgery has challenged the traditional administration of large amounts of fluids during surgery[108,109].

These findings have prompted fervent discussion on how liberal or restrictive perioperative fluid management should be applied, and several randomised controlled trial have attempted to settle this issue[104,108,110,111].

Due to the lack of consensus on the optimal implementation of fluid management, a new and more precise approach based on goal-directed fluid therapy and individualised fluid administration has been developed[103]. Goal-directed fluid optimisation has markedly increased tissue oxygen tension and microcirculatory perfusion in both healthy and perianastomotic tissues compared to the restricted fluid strategy[106,112,113].

Central venous pressure (CVP) remains the most

widely used clinical marker of volume status, despite numerous studies indicating no association between CVP and circulating blood volume[114]. Because of this limitation, central venous and pulmonary artery occlusion pressures, which are the only variables for guided fluid therapy and optimised preload, are not recommended. Dynamic parameters such as stroke volume variation or pulse pressure variation provide a more favourable prediction of fluid responsiveness. Individualised goal-directed fluid therapy, particularly oesophageal Doppler-guided fluid optimisation, has been shown to improve patient outcomes and to reduce the length of hospital stays compared with conventional fluid replacement[115]. Doppler-guided fluid boluses appear to improve clinical outcomes, particularly in elderly and frail patients[116,117]. This method, however, cannot be universally performed for practical and financial reasons[118].

Using a "goal-directed" approach, it is generally possible to replace lost plasma, whereas the extracellular compartment cannot currently be monitored. Therefore, losses from the latter should be replaced based on the protocol suggested by Chappell *et al*[101], which involves the substitution of insensible perspiration with 1 mL/kg per hour during abdominal surgery and does not include the possibility of primary fluid consumption by the third space, the existence of which is denied[119].

The optimal solution for volume replacement and optimisation remains an ongoing issue of heated debate. The goal of perioperative fluid management is to maintain fluid balance and to minimise the possible risks by choosing the right fluid at the right time.

Colloids are criticised because of their ability to diffuse into the interstitium, making further extravasation more likely[120], because of the cumulative and persistent effects related to their infusion[121] and, finally, because of safety concerns. Recent studies of the potential increase in the risk of bleeding and acute kidney injury following the application of various colloids have shown that the use of hydroxyethyl starch appears to be associated with an increased need for dialysis[122] and might even increase mortality in patients with sepsis[123].

Current evidence suggests that beyond fluid composition, the timing and volume of the administered fluid represent two additional factors that are likely to influence perioperative patient outcome. For patients with mild-to-moderate volume deficits, crystalloids are still the first choice. In the case of severe volume depletion, we recommend starting fluid resuscitation with a colloid to rapidly reverse volume deficits and ensure oxygenation and then to switch to crystalloids once the patient approaches euvolaemia.

Goal-directed fluid management enables appropriate use of fluids, vasopressors, and inotropes, and results in improved outcomes. The vasodilatatory effect of anaesthetic cannot be ignored and must be expected to terminate at the end of surgery. Treating vasodilatation with crystalloids or colloids can be a mistake in all euvolaemic patient, whereas vasopressor infusion during surgical op-

eration can help in avoiding excessive fluid overload[124,125].

Optimisation of intraoperative ventilation

Postoperative pulmonary complications following major upper abdominal surgery increase morbidity, mortality, the length of hospital stay and costs[33]. Reduced lung inflation represents one of the basic mechanisms of postoperative pulmonary complications. The adjustment of the body positioning from upright to supine itself can reduce the resting lung volume by approximately 0.8-1.0[126]. The additive effect of supine positioning, general anaesthesia, and abdominal incisions significantly reduces functional residual capacity and increases airway resistance. In addition, during the induction of anaesthesia, most of the general anaesthetics further reduce functional residual capacity. The combination of these effects predisposes patients to atelectasis with the risks of hypoxemia and infection. Additionally, postoperative pain and the use of analgesics can contribute to a reduced tidal volume and impaired clearing of secretions, depending on adequate coughing and deep breathing[126,127].

Mechanical ventilation is mandatory in patients undergoing general anaesthesia. High tidal volumes can overdistend non-injured lungs, particularly in non-dependent lung tissues. The non-aerated atelectatic lung regions are prone to repeated collapse and re-expansion of the alveoli, causing shear stress and diffuse mechanical damage of the alveoli. During surgical procedures, both phenomena can induce stress in non-injured lung tissues, triggering local inflammation[128,129]. Retrospective and prospective studies have shown the potential beneficial effects of reduced tidal volumes in patients who are on short-term mechanical ventilation following surgery[130]. Protective mechanical ventilation using reduced tidal volumes can accordingly reduce ventilator-associated lung injury. The application of positive end expiratory pressure (PEEP) can prevent alveolar collapse and atelectasis formation, and recruitment manoeuvres can support the beneficial effects of PEEP during short-term ventilation[131]. Effective anaesthesiological management during pancreatic surgery should involve the application of a protective ventilation strategy (lower tidal volumes < 8 mL/kg, PEEP = 6-12 mmHg and recruitment manoeuvres) to improve respiratory function during the postoperative period following abdominal surgery and to reduce the clinical signs of pulmonary infection during the postoperative period[132].

Intraoperative thromboprophylaxis

The use of LMWHs to prevent thrombotic events in these patients represents a common and well-documented practice. Effective pharmacological thromboprophylaxis includes the administration of LMWH from the day prior to the surgery. In addition to this useful approach mechanical prophylaxis including graduated compression stockings and intermittent pneumatic compression is highly recommended during the surgical operation and during the postoperative period until the risk of bleeding

has diminished and the application of new pharmacological prophylaxis might be initiated[57,60].

Thromboelastography can play a potential role, despite its limitations, as a valuable tool for the evaluation of the entire perioperative coagulation process and hypercoagulability changes, as well as for increasing patient safety through more effective management of antithrombotic therapy[133,134].

POSTOPERATIVE MANAGEMENT

Over the past 20 years, surgery and anaesthesia for patients undergoing abdominal surgery have undergone immense development. A novel concept of perioperative patient care following surgical abdominal procedures has emerged. Fast track programmes, a new concept of enhanced recovery after surgery and the implementation of multimodal rehabilitation, have heavily influenced this modern change, optimising perioperative care, accelerating recovery and reducing hospital stays and costs. The objective of this integrated approach between surgeon, anaesthetist, nurses and physiotherapist is to reduce the impact of surgery on patient homeostasis. The main pillars of this new management are those shared by fast track surgery and can be summarised as follows: (1) reduction of surgical invasiveness (early removal of drains, nasogastric tube, small incisions, pharmacological stimulation of the gut); (2) pain relief/non-opioid oral analgesia; (3) early oral nutrition/goal-directed fluid therapy; (4) intensive postoperative ambulation and prevention of venous thromboembolism; and (5) intensive respiratory rehabilitation.

All of these basic points, combined with the prevention of intraoperative hypothermia, neural blockades[135], maintenance of euglycaemia, and the development of goal-directed fluid therapy contribute to the reduction of surgical stress.

A systematic review of the literature regarding perioperative care in pancreatic cancer surgery has revealed a limited number of studies providing low levels of evidence[50,54,136]. Despite their potential weaknesses, the studies detailed above have demonstrated that implementation of fast-track peri-operative care pathways is feasible in pancreatic surgery and can be associated with reduced length of stay, reduced relevant hospital costs and no increase in morbidity, 30-d mortality or re-admission rates.

Early nasogastric tube, catheter and drain removal
Nasogastric tube: Nasogastric tubes have been routinely used following abdominal surgery until normal bowel function is restored, following the notion that gastric decompression resulting from decreased air and fluid accumulation can prevent abdominal distension, nausea and vomiting. Many studies have subsequently questioned this practice, advising against its routine use. In fact, prophylactic nasogastric tube aspiration is associated with pulmonary complications[137] and significant patient discomfort. A recent study on the implementation of fast-track recovery pathways in pancreatic surgery[138] has

underlined the advantages of the early removal of nasogastric tubes and early oral feeding in terms of incidence of delayed gastric emptying and earlier bowel activity. Given the risk of pulmonary complications, significant patient discomfort and lack of benefit associated with prophylactic nasogastric tube aspiration, this practice should not be routinely used[139,140].

Consistent with a recent study, in our daily practice, we remove nasogastric tubes on postoperative day 1 only if the tube drainage amount is less than 300 mL or at the end of surgery in cases of distal pancreatectomy which makes delayed gastric emptying less frequent[52].

Abdominal drains: The presence of an abdominal drain represents a significant impediment to achieving early and appropriate levels of mobilisation. Several randomised trials have not found any benefit of prophylactic drains after surgical operations, such as cholecystectomy[141], colorectal surgery[142] or hepatectomy[143]. Rather, these prospective randomised studies found that routine drainage resulted in an increased frequency of complications and no difference in outcome.

Because pancreatic surgery is associated with high rates of morbidity, the purpose of prophylactic drainage is to prevent fluid collection and to aid in the early detection of anastomotic leak and associated haemorrhage. Following pancreatectomy, the use of a prophylactic drain is supported by the belief that the early detection of pancreatic fistulae through the measurement of amylase in the draining fluid will allow for the efficient management and the avoidance of major complications[144]. Despite reports of randomised, control trials and cohort studies that do not support the use of drains, most surgeons routinely place prophylactic intraperitoneal drains at the time of pancreatic resections[145,146]. Evidence-based practice guidelines for drain management during pancreatectomy remain to be established despite the remarkable number of studies that are available to help guide practice.

At our University Hospital, abandoning drainage during pancreatic surgery is believed to be unsafe, and according to Kaminsky *et al*[146], it is reasonable to suggest a practice of selective drainage based on the presence of risk factors. The presence of soft pancreas texture, a small pancreatic duct diameter, increased intraoperative blood loss (> 200 mL) and prolonged operative time are risk factors that reflect abdominal drains. In the case that patient is doing well and the drain amylase levels are below 5000 U/L, drains [on postoperative day 1 (POD 1)] can be safely removed on POD 3 in patients with low risk of pancreatic fistulae.

Early oral nutrition
The restoration of normal gastrointestinal function to allow adequate food intake and rapid recovery is one of the primary objectives of postoperative care. A meta-analysis of controlled trials of early enteral or oral versus 'nil by mouth' feeding after gastrointestinal surgery indicated no clear advantage to continued patient fasting after the elec-

tive gastrointestinal resection[147].

Concerning nutrition, studies have clearly found that allowing eating/drinking until late the day prior to surgery and commencement of eating/drinking soon after surgery has many advantages[148,149]. Through the earlier intake of fluids and solids, the gastrointestinal system is less affected with an earlier initiation of normal intestinal activity.

An interesting review analysing which feeding routine was more favourable following pancreatoduodenectomy revealed no consensus in terms of postoperative nutrition of patients who had undergone pancreatic surgery. Current European guidelines recommend routine enteral feeding after pancreatoduodenectomy, whereas the American guidelines do not. Gerritsen *et al*[150] concluded that there is no evidence to support routine enteral or parenteral feeding after pancreatoduodenectomy, whereas the oral diet appears to be the best feeding strategy.

At our University Hospital, it is common to allow the patient to take clear liquids from POD 1 but not before 6 h postoperatively and a light diet from POD 2, in the absence of any complications. In patients at risk of postoperative complications such as pancreatic fistulae or abdominal collections, we advocate the use of combined parenteral and enteral nutrition[52].

Total pancreatectomy and postoperative glycaemic control

Total pancreatectomy, usually performed for the treatment of multifocal disease or in case of atrophic, soft, friable remnant pancreatic tissue is responsible of endocrine and exocrine insufficiency. In addition to the absence of insulin, the endocrine abnormalities accompanying total pancreatectomy include both glucagon and pancreatic polypeptide deficiencies, which appears to play a key role in the increased hepatic insulin resistance observed in pancreatogenic diabetes[151]. Moreover, following pancreatectomy, insulin receptors are upregulated peripherally, rendering patients uniquely sensitive to hormone replacement[152].

This type of diabetic condition is defined "pancreatogenetic" diabetes and is often considered to be different from type 1 and 2 diabetes. This diabetic state is commonly described as "brittle", as a result of enhanced peripheral insulin sensitivity, decreased hepatic insulin sensitivity and reduction of glucagon secretion. The resulting labile glycaemic control is characterized by periodic episodes of both hyper and hypoglycaemia[153,154].

In recent years, studies have shown that diabetes following total pancreatectomy is not necessarily associated with poor glycaemic control, and the majority of cases exhibit equivalent biochemical controls compared to the normal type 1 diabetic population[155,156].

Recently, the development of accurate, continuous blood glucose monitoring devices, particularly closed-loop systems, for computer-assisted blood glucose control in the intensive care unit have been reported to assist in obtaining favourable glycaemic control in patients with pancreatogenic diabetes following pancreatic resection[157].

The hyperglycaemia induced by surgical stress can-

not be controlled using the conventional sliding scale method[158], whereas the perioperative use of an artificial endocrine pancreas enables strict glycaemic control of euglycaemia without severe hypoglycaemia[159,160].

Modern pancreatic enzyme formulations have improved exocrine insufficiency, facilitating glycaemic control due to the avoidance of malabsorption[155].

The enhanced patient understanding of the consequences of total pancreatectomy, early education on diabetes (all patients should consult an endocrinologist immediately following their operation), advances in medical therapies, and blood glucose monitoring might all have contributed to enhanced glycaemic control[161].

Goal-directed fluid therapy

Early oral nutrition has to be associated to the individualised postoperative fluid therapy that is administered in accordance to the optimisation of stroke volume. Dynamic parameters such as stroke volume or pulse pressure variation can provide a more favourable prediction of fluid responsiveness. Oesophageal Doppler-guided fluid optimisation has been shown to improve patient outcomes, although this method cannot be performed on conscious patients[116,117]. Fluid challenges and the leg-raising test can represent simple and valid alternatives[118]. Thus, oral nutrition has clearly be associated with a progressive decrease of intravenous fluids.

Pain relief/non opioid oral analgesia

One aim of fast track surgery is to obtain favourable pain control, which is intended to enable patient mobilisation, coughing and early nutrition. One of the modern principles for analgesia is the concept of opioid-sparing, which enhances recovery by avoiding the opioid-related side effects. In major abdominal procedures, the administration of continuous thoracic epidural analgesia with local anaesthetics has been demonstrated to be the most efficient technique to obtain optimal analgesia, allowing for early mobilisation, reducing postoperative ileus and pulmonary morbidity[162], and therefore acting as an important component of multimodal recovery strategies[163,164]. A midthoracic epidural activated prior to the initiation of surgery also blocks stress hormone release[165] and attenuates postoperative insulin resistance[166,167].

Fast-track clinical pathways in the peri-operative care of patients undergoing pancreatic resection provide for a catheter placed in the midthoracic level at T8/9 to achieve both analgesic and sympathetic blocks[168].

Small doses of epidural opioids have been shown to act in synergy with epidural local anaesthetics in providing analgesia, allowing reduced dosages of both agents[169].

For break-through pain, non-steroidal anti-inflammatory drugs and bolus epidural bupivacaine should be administered whilst the epidural is running. Non-steroidal anti-inflammatory drugs should be administered just prior to the removal of the epidural and continued until and/or after discharge.

As the optimal duration of continuous postoperative

mid-thoracic epidural analgesia has not been established in well-designed randomised trials, we suggest that two-to-three days might be a sufficient period for pancreatic surgery.

Patient-controlled analgesia using intravenous opioids does not provide the same efficient analgesia and elicits less beneficial physiological effects on surgical stress responses compared to local epidural anaesthetic techniques. However, it is performed whenever contraindications prevent the execution of peridural analgesia.

Intensive postoperative ambulation and prevention of venous thromboembolism

Among the standardised clinical pathways, which represent the basis of the fast-track programme, early mobilisation is a cornerstone. It has been shown to play a major role in postoperative functional recovery. Improved early ambulation can elicit beneficial effects in the resolution of postoperative ileus and can reduce the risk of lower extremity deep venous thrombosis. Furthermore, mobilisation might reduce pulmonary complications[170]. The risk for VTE, which is particularly high in this patient population, must be managed from the beginning of the preoperative period and continue during the entire surgical operation until the postoperative period as a result of early mobilisation and proper pharmacological thromboprophylaxis. At our University Hospital, we generally mobilise patients out of their beds for more than one hour from POD 1 and progressively increase the hours of mobilisation from POD 2. Patients who had undergone major abdominal surgery for gastrointestinal malignancies should be considered for post-discharge VTE prophylaxis for up to 4 wk following surgery during the following situations: residual or metastatic disease, obesity or previous history of VTE.

Intensive respiratory rehabilitation

Pulmonary complications following pancreatic resection occur in approximately one quarter of all patients[171]. Many pathophysiological modifications that occur under anaesthesia and/or following surgery can interact with each other, resulting in respiratory complications.

Reduced lung inflation is one of the basic causes of postoperative pulmonary dysfunction[172].

After upper abdominal and thoracic surgery, postoperative diaphragmatic dysfunction[173], which is the most important determinant of respiratory complications and atelectasis, is commonly observed and is caused by the mechanical compression of alveoli and the resorption of alveolar gases, which are the factors most commonly implicated in respiratory complications[174].

In recent years, breathing (deep breathing and directed cough) and chest wall physiotherapy have been introduced into clinical practice to prevent pulmonary complications. Physiotherapy includes a variety of manual treatments (postural drainage, percussion, clapping, vibration, or shaking) as well as the use of mechanical breathing devices (incentive spirometry, blow bottles, intermittent positive pressure breathing, and continuous positive airway pressure).

A systematic review showed that postoperative non-invasive ventilation, specifically continuous positive airway pressure (CPAP), improves hypoxaemia and reduces both postoperative complications and the requirement for intubation in patients undergoing abdominal surgery[170]. Furthermore, there is no specific study focusing on the role of chest physiotherapy after pancreatic resection; it is nonetheless included in the care plan at our institution. Every patient who has undergone pancreatic surgery is instructed to use a blow bottle (5 min/h) and undergoes an individualised exercise schedule that is designed by physiotherapists. Further, certain short courses of non-invasive mechanical ventilation (CPAP) can be performed as needed.

Intensive postoperative management

Despite continuous improvements in operative technique and perioperative management, the increasing age of patients undergoing major abdominal surgery exposes patients to an increasing number of postoperative complications, leading to increased morbidity, mortality, length of hospital stay, and hospital costs. Although the concept of fast-track surgery has questioned the traditional use of intensive care units, there is increasing evidence indicating that access to ICUs results in a more favourable impact on the outcomes of major abdominal surgeries.

In the case of pancreaticoduodenectomy, even high-volume centres report a major postoperative complication rate of approximately 20%[175]. Because of these observations, patients who undergo pancreatic cancer surgeries might benefit from admission to the ICU.

An ideal ICU model should involve the cooperation of the intensivists who primarily care for the patients with the primary physician and surgeon[176].

Current general concepts of fast track surgery have been implemented in intensive care units. Early mobilisation, early enteral feeding, and restrictive perioperative fluid management are generally performed at the ICUs of our institution. In addition to these programmes, ICU stays can offer extended haemodynamic monitoring, which is useful in goal-directed fluid therapy, the possibility of invasive and non-invasive ventilation, the continuous application of intravenous drugs or subsequently required extracorporeal procedures.

In summary, most patients who undergo elective pancreatic surgery for cancer do not necessary require intensive care admission, whereas high-risk patients might benefit from postoperative care in the ICUs. We suggest that surgical intensive care units play a pivotal role in the perioperative care of patients undergoing major abdominal surgeries, and patients with co-morbidities or elderly patients should be scheduled for intensive care treatment[177,178].

CONCLUSION

In recent decades, diagnostic modalities and the surgical treatments of PC have significantly progressed, de-

spite the fact that overall prognosis has only marginally changed. The management of patients affected by PC is complex and requires expertise in many fields. Multidisciplinary teams are necessary to optimise and improve the overall care and outcomes of patients. Because more patients are referred to surgery at an advanced age, a coordinated effort between surgeon and anaesthetist in terms of risk assessment is necessary, particularly for borderline resectable or unresectable disease cases (to spare the risk and cost of surgery for patients who are affected by advance disease and whose life expectancy might be potentially shortened by an unuseful and dangerous surgical operation)[179]. More favourable outcomes are attained if PC patients are appropriately referred to tertiary centres for assessment by surgical, medical and radiation oncologists, gastroenterologists, anaesthetists and other dedicated health care providers. The anaesthetist plays a key role in the preoperative assessment, intraoperative management and during the postoperative period assessment. For this reason, close cooperation between surgeons and anaesthesiologists is crucial for ensuring the safe performance of major gastrointestinal surgery with acceptable morbidity and mortality rates.

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2014 ADVANCES IN PANCREATIC CANCER

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Involvement of substance P and the NK-1 receptor in pancreatic cancer

Miguel Muñoz, Rafael Coveñas

Miguel Muñoz, Virgen del Rocío University Hospital, Research Laboratory on Neuropeptides (IBIS), 41013 Seville, Spain

Rafael Coveñas, Institute of Neurosciences of Castilla y León (INCYL), Laboratory of Neuroanatomy of the Peptidergic Systems (Lab 14), University of Salamanca, 37007 Salamanca, Spain

Author contributions: Muñoz M and Coveñas R contributed equally to this work.

Correspondence to: Dr. Miguel Muñoz, Virgen del Rocío University Hospital, Research Laboratory on Neuropeptides (IBIS), Unidad de Cuidados Intensivos Pediátricos Av. Manuel Siurot s/n, 41013 Seville, Spain. mmunoz@cica.es

Telephone: +34-95-5012965 Fax: +34-95-5012921

Abstract

Pancreatic cancer is the fourth leading cause of cancer related-death for both men and women and the 1- and 5-year relative survival rates are 25% and 6%, respectively. Thus, it is urgent to investigate new antitumor drugs to improve the survival of pancreatic cancer patients. The peptide substance P (SP) has a widespread distribution throughout the body. After binding to the neurokinin-1 (NK-1) receptor, SP regulates biological functions related to cancer, such as tumor cell proliferation, neoangiogenesis, the migration of tumor cells for invasion, infiltration and metastasis, and it exerts an antiapoptotic effects on tumor cells. It is known that the SP/NK-1 receptor system is involved in pancreatic cancer progression: (1) pancreatic cancer cells and samples express NK-1 receptors; (2) the NK-1 receptor is overexpressed in pancreatic cancer cells in comparison with non-tumor cells; (3) nanomolar concentrations of SP induce pancreatic cancer cell proliferation; (4) NK-1 receptor antagonists inhibit pancreatic cell proliferation in a concentration-dependent manner, at a certain concentration, these antagonists inhibit 100% of tumor cells; (5) this antitumor action is mediated through the NK-1 receptor, and tumor cells die by apoptosis; and (6) NK-1 receptor antagonists inhibit angiogenesis in pancreatic cancer xenografts. All these data suggest that the SP/NK-1 receptor system could play an important role in the development of pancreatic cancer; that the NK-1 receptor could be a new promising therapeutic target in pancreatic cancer, and that NK-1 receptor antagonists could improve the treatment of pancreatic cancer.

Core tip: The substance P (SP)/neurokinin-1 (NK-1) receptor system plays an important role in pancreatic cancer progression. Pancreatic cancer cells overexpress NK-1 receptors and SP promotes angiogenesis and the proliferation and the migration of pancreatic tumor cells. By contrast, NK-1 receptor antagonists, in a concentration-dependent manner, inhibit pancreatic cell proliferation (tumor cells die by apoptosis), have antiangiogenic properties in pancreatic cancer and block the migratory activity of pancreatic tumor cells. The antitumor action is mediated through the NK-1 receptor. Thus, the NK-1 receptor could be a new promising therapeutic target in pancreatic cancer and NK-1 receptor antagonists could improve pancreatic cancer treatment.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related-death for both men and women, with less than 5% survival at 5 years after diagnosis. In 2013, the American Cancer Society estimated 45220 new cases of pancreatic cancer in the United States and 38460 deaths from the disease. Treatment strategies have not succeeded in significantly extending patient survival, and neither have clinical outcomes improved substantially over the past 35 years; the overall 5-year survival rate remains dismal, at around 5%[1]. Pancreatic cancer remains a major unsolved health problem and conventional treatments having little impact on the course of the disease. Moreover, almost all patients with pancreatic cancer develop metastases, this being the primary reason for its lethality[2]. Accordingly, there is an urgent need to improve current therapies. Cytostatic drugs show a low safety profile and severe side effects, since they are not specific to tumor cells. Research should focus on drugs with the same or greater antitumor action but with fewer side effects. This can only be achieved if the drug is specific against pancreatic cancer cells and researchers are therefore seeking to identify novel molecular targets for blocking pancreatic cancer growth.

For some years, the expression and secretion of peptides by tumors has attracted increasing interest[3]. Substance P (SP) is an undecapeptide that is widely distributed throughout the body. It is derived from the pre-protachykinin A gene and belongs to the tachykinin family of peptides. The biological actions of tachykinins (SP, neurokinin A, neurokinin B...) are mediated through the neurokinin-1 (NK-1), NK-2 and NK-3 receptors. SP has the highest affinity for the NK-1 receptor, which shows a widespread distribution throughout the body. This means that the biological actions (*e.g.*, pain, neurogenic inflammation, regulation of the cardiovascular system, mitogenesis...) exerted by the SP are mainly mediated by the NK-1 receptor[4,5]. Moreover, there are many data suggesting the involvement of the SP/NK-1 receptor system in cancer[5] (Figure 1 and Table 1). SP and NK-1 receptors have been detected in tumor cells and in intra- and peri-tumoral blood vessels[4-6]. SP induces mitogenesis in normal and tumor cells, protecting the latter from apoptosis, and controls the migration of tumor cells[4,7,8]. This is extremely important since the prevention of metastasis is a major goal in the treatment of tumors because over 90% of cancer deaths are derived not from the primary tumor but from the development of metastases. Moreover, it has recently been reported that the extravasation of tumor cells into the brain to form cerebral metastases may be an SP-mediated process[9]. More specifically, it has been reported that the SP/NK-1 receptor system is involved in pancreatic cancer by inducing pancreatic cancer proliferation, neoangiogenesis, and migration of pancreatic cancer cells (invasion, infiltration and metastasis). By contrast, NK-1 receptor antagonists inhibit pancreatic cancer cell proliferation (tumor cells die by apoptosis), angiogenesis and the migration of pancreatic cancer

Table 1 Technical features of NK-1 receptor antagonists	
NK-1 receptor antagonists	**Feature**
Therapeutic action	Linked to stereochemical features (receptor affinity) and not to chemical composition
Cell specificity	Specific cytotoxicity against pancreatic cancer cells *via* the NK-1 receptor
Antitumor action	Mitogenesis inhibition
	Cell death by apoptosis
	Angiogenesis inhibition
	Inhibition of the migration of cancer cells: Inhibit invasion, infiltration and metastasis
Beneficial effects	Central nervous system:
	Antiemetic
	Anxiolytic
	Antimigraine
	Anticonvulsant
	Neuroprotector
	Peripheral nervous system:
	Neuroprotector
	Liver:
	Hepatoprotector
	Kidney:
	Nephroprotector
	Systemic:
	Analgesic
	Antiinflammatory
	Antiviral
Side-effects	Headaches, hiccupping, vertigo and drowsiness
Synergistic effect with cytostatic and radiation therapy	Vinblastine, adriamycin, mitomycin, ifosfamide, cisplatin
Decrease cytostatic and radiation therapy side-effects	Cisplatin, cyclophosphamide
Block multiple intracellular signaling pathways	NK-1 receptor (G protein-coupled receptor): Rho-Rock-pMLC: Cell migration inhibition
	PLC-IP$_3$-Akt: Apoptotic effect
	PLC-DAG-TK-MAPKs: Inhibition of tumor cell proliferation
	PLC-DAG-PKC-MAPKs: Inhibition of tumor cell proliferation
	ATP-cAMP-PKA-Phosphorylation
	PLA-Arachidonic acid-PGs
	TXAs
	LXs
	Glycogen breakdown inhibition (counteract the Warburg effect)
Dosage	Act at μmol/L in a concentration-dependent manner

Akt: Protein kinase B; ATP: Adenosine triphosphate; cAMP: Cyclic adenosine monophosphate; DAG: Diacilglicerol; IP3: Inositol triphosphate; LXs: Leukotrienes; MAPKs: Mitogen-activated protein kinase; PGs: Prostacyclin; PKA: Protein kinase A; PKC: Protein kinase C; PLA: Phospholipase A; PLC: Phospholipase C; pMLC: Myosin regulatory light chain phosphorylation; TK: Tyrosine-kinase; TXAs: Thromboxanes.

cells[10-14] (Figure 1 and Table 1).

In sum, all the data reported above suggest that novel possibilities for translational research are emerging to improve the diagnosis and treatment of pancreatic cancer. Here, we review the involvement of the SP/NK-1 receptor system in pancreatic cancer and, specifically, the use of NK-1 receptor antagonists as antitumor drugs in pancreatic cancer (Figure 1 and Table 1).
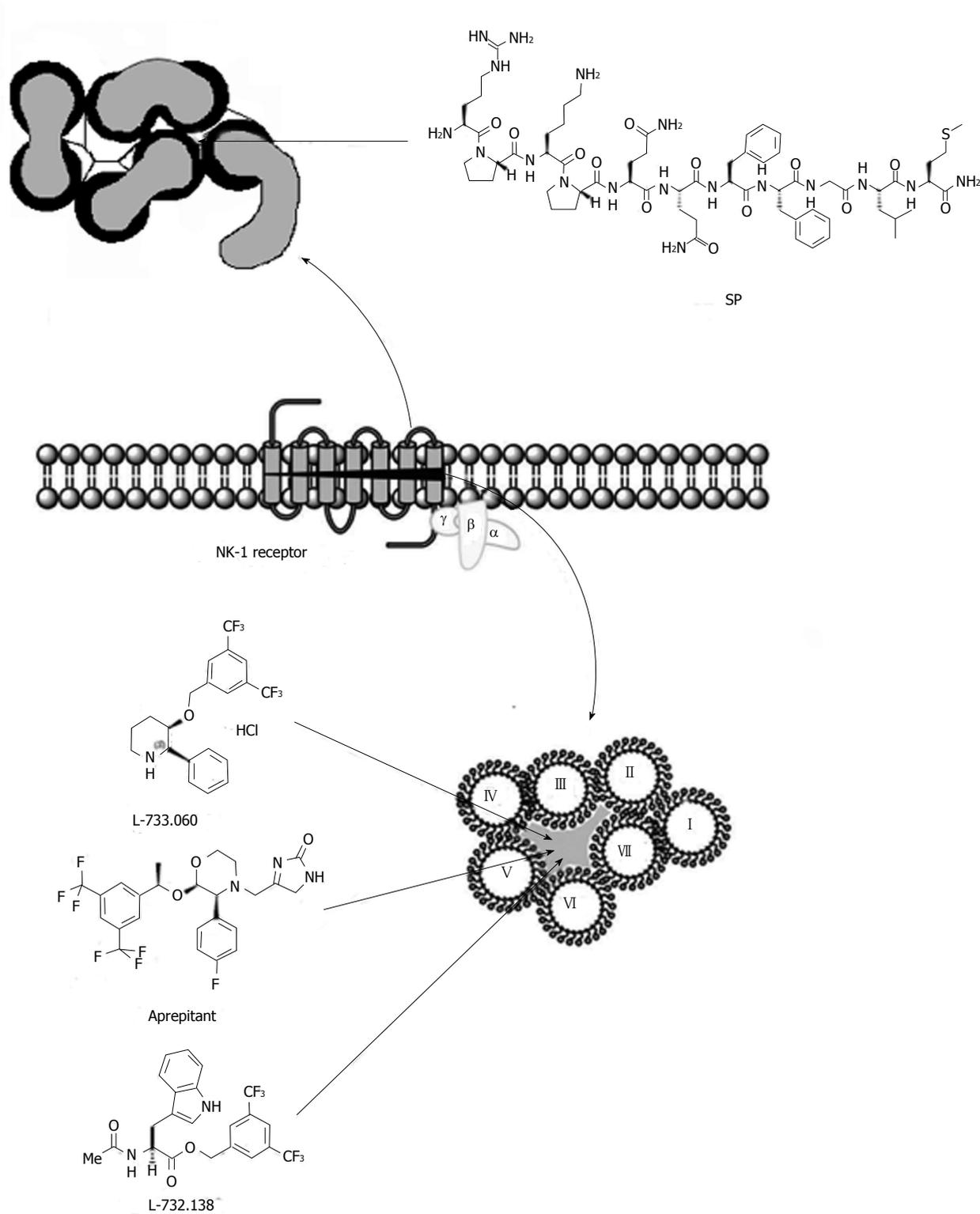
Figure 1 Substance P and neurokinin-1 receptor antagonists bind to different sites of the neurokinin-1 receptor. Substance P (SP) binds to the extracellular loops of the receptor, whereas neurokinin-1 (NK-1) antagonists (*e.g.*, L-733.060, aprepitant, L-732.138) bind more deeply, between the transmembrane segments.

PANCREATIC CANCER CELLS AND SAMPLES EXPRESS NK-1 RECEPTORS

The NK-1 receptor is synonymous with the SP receptor and tachykinin receptor 1. The NK-1 receptor is a G protein-coupled receptor (GPCR) that mediates the action of SP and other tachykinins[15,16]. The NK-1 receptor consists of 407 amino acid residues; it has a molecular weight of 58 kDa, and it is made of seven hydrophobic transmembrane domains with three extracellular and three intracellular loops, an amino-terminus and a cytoplasmic carboxy-terminus[17,18] (Figure 1). The loops have functional sites, including two cysteines amino acids for a disulfide bridge, Asp-Arg-Tyr, which is responsible for

the association with arrestin and, Lys/Arg-Lys/Arg-X-X-Lys/Arg, which interacts with G-proteins[18,19]. The NK-1 receptor is coupled to the Gq family of G proteins and its activation leads to the hydrolysis of membrane phosphoinositides, resulting in the formation of two-second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)[20,21]. The formation of IP3 triggers the release of calcium from intracellular stores and the formation of DAG leads to the activation of protein kinase C. Together, these messengers cause a cascade of protein phosphorylation/dephosphorylation reactions, culminating in altered gene expression and cell function.

SP is an undecapeptide widely distributed throughout the body and it is the natural ligand showing the highest affinity for the NK-1 receptor (Figure 1). In fact, the NK-1 receptor has been defined as a mediator of the biological activities encoded by the C-terminal sequence of tachykinins, for which SP is a more potent agonist than neurokinin A or neurokinin B[22]. After binding to the NK-1 receptor, SP regulates many biological functions (*e.g.*, pain, neurogenic inflammation, mitogenesis...)[4,5], although other NK receptors could also be involved (*e.g.,* NK-2) in these actions. After the binding of SP to the NK-1 receptor, both are internalized into endosomes; the undecapeptide induces a clathrin-dependent internalization of the receptor, after which SP is degraded and the NK-1 receptor is recycled to the cell surface[23-26]. SP-NK-1 receptor binding can generate second messengers [cyclic adenosine monophosphate (cAMP) accumulation *via* stimulation of adenylate cyclase; stimulation, *via* phospholipase C, of phosphatidyl inositol turnover, leading to calcium mobilization; arachidonic acid mobilization *via* phospholipase A2], triggering numerous effectors mechanisms involved in cellular excitability and in the regulation of cell function[4,5,27].

It is known that pancreatic cancer cells and samples express the NK-1 receptor[10,13,14]. This receptor has been also demonstrated in human cancer cell lines and/or in primary tumors (*e.g.*, glioma, astrocytoma, retinoblastoma, ganglioneuroblastoma, leukemia, neuroblastoma, carcinomas (larynx, gastric, colon, medullary thyroid, breast, oral...)[4-6,10,28-37]. In addition, in most tumors investigated NK-1 receptors have been found in intra- and peritumoral blood vessels. This is quite important regarding the involvement of the NK-1 receptor in angiogenesis[6]. NK-1 receptors have been located in both the plasma membrane and the cytoplasm of tumor cells and, occasionally, in the nucleus of these cells[31,34,38]. Moreover, several isoforms (33-38, 46, 54-58 and 75 kDa) of the NK-1 receptor have been reported in human cancer cells (*e.g.*, neuroblastoma, retinoblastoma, larynx carcinoma, gastric adenocarcinoma, leukemia, *etc*.)[33-36,38]. Regarding the pancreatic cancer, it has been reported that its tumor cells express several isoforms (36, 46, 58 and 75 kDa)[10,13,14]. However, in order to clarify the functional roles of these isoforms, further research is needed. In humans, the presence of two subtypes of the NK-1 receptor has been reported: the full-length one and the truncated one. The former mediates a slow growth of tumor cells and the second enhances the growth of these cells to a considerable extent and stimulates the production of cytokines with growth-promoting functions[39]. It seems that these cytokines activate a transcription factor (NF-κB) that upregulates the truncated NK-1 receptor form and slightly increases the full-length form[40,41]. It is also known that the truncated form, an oncogenic isoform of the NK-1 receptor, mediates malignancy in tumor cells[39] and that the truncated NK-1 receptor is increased in colonic epithelial cells from patients with colitis-associated cancer[42].

In the first study in which NK-1 receptors were reported in pancreatic cancer (1 of 9 samples)[6], the authors applied an autoradiographic method. Later, in another study, NK-1 receptor expression was reported in 27% of the samples[43]. However, a third study compared 50-pancreatic human cancer samples obtained from pancreatoduodenoctomy (Whipple operation) with normal controls[10]. In these cases, the authors found the expression of NK-1 receptors in all the pancreatic cancer samples. Thus, by using *in situ* hybridization and immunohistochemistry techniques, in normal pancreas NK-1 receptor mRNA and NK-1 receptor immunoreactivity were occasionally weakly observed in acinar and ductal cells, but a moderate to strong NK-1 receptor mRNA signal and NK-1 receptor immunoreactivity were present in most of the cancer cells[10]. Moreover, the growth of the tumor mass, peritumoral infiltration and metastasis could be regulated by the SP/NK-1 receptor system, overexpressed in tumor cells and in tumoral and peritumoral tissue in pancreatic cancer (inflammatory cells, fibroblasts, blood vessels, nerves, ganglia, islet)[10].

The NK-1 receptor is also known to be involved in the viability of tumor cells. It has been reported that after a knockdown gene-silencing method (siRNA), the NK-1 receptor is involved in the viability of such cells[33,34,37]. Following the administration of the siRNA *TACR1* (tachykinin 1 receptor gene) to cultured tumor cells, more apoptotic cells were found in siRNA cells than in cells not transfected, and hence the number of siRNA tumor cells was significantly decreased in comparison with the number of non-transfected cells[33,34,37].

NK-1 RECEPTOR IS OVEREXPRESSED IN PANCREATIC CANCER CELLS IN COMPARISON WITH NON-TUMOR CELLS

It is known not only that the NK-1 receptor is expressed in tumor cells, but also that this receptor is overexpressed in such cells (*e.g.*, glioblastoma, breast cancer, retinoblastoma, larynx, pancreatic, gastric and colon carcinomas...)[4,5,10,30,33,34,37]. This is important, since the visualization of NK-1 receptors by immunohistochemistry for diagnostic or therapeutic purposes would facilitate the identification of tumors overexpressing this receptor[44]. It is known that normal cells express a lower number of NK-1 receptors than tumor cells (*e.g.*, human pancreatic cancer cell lines express more NK-1 receptors than

control cells)[10]; that tumor samples from patients with advanced tumor stages exhibit significantly higher NK-1 receptor levels[10]; that TACR1 mRNA is present in human acute lymphoblastic leukemia cell lines, with the highest levels in these cells and the lowest ones in normal cells[33]; that astrocytoma/glioma cell lines in culture shows a lower number of NK-1 receptors than astrocytoma/glioma primary tumors; that glioblastomas express more NK-1 receptors than astrocytomas, and that the most malignant phenotypes of tumors show a higher rate of NK-1 receptor expression and are associated with advanced tumor stages and a poorer prognosis[6,10,45]. The data suggest that the number of NK-1 receptors could be correlated with the degree of malignancy. Thus, the overexpression of the NK-1 receptor in tumor cells suggests the possibility of finding a specific treatment against cancer using NK-1 receptor antagonists and, in this way, the side effects of the treatment could be decreased considerably. This strategy opens up new approaches for cancer treatment. Moreover, following the use of real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology in 50 pancreatic human cancer samples obtained from pancreatoduodenoctomy (Whipple operation), NK-1 receptor mRNA levels were increased 36.7-fold in these samples in comparison with normal controls. Enhanced NK-1 receptor expression levels were not related to tumor grade but were associated with advanced tumor stage and a poorer prognosis. As reported above, NK-1 receptor mRNA levels and NK-1 receptor immunoreactivity are higher in human pancreatic cancer samples than in normal pancreas[10]. Moreover, using a Western blot analysis, the NK-1 receptor was found to be increased 26-fold in pancreatic cancer samples in comparison with normal controls. NK-1 receptor mRNA was detected in five pancreatic cancer cell lines by real-time quantitative RT-PCR, the highest levels being observed in CAPAN-1 cells and the lowest ones in ASPC-1 cells. SP and SP analog agonists stimulated pancreatic cancer cell growth, depending on the NK-1 receptor expression level, and this effect could be blocked by a selective NK-1 receptor antagonist in a concentration-dependent manner[10,13].

It has been suggested that chronic inflammation could be correlated with an increased risk of developing cancer. It is known that the risk of pancreatic cancer is very high in subjects with chronic pancreatitis and appears to be independent of sex, country, or type of pancreatitis[46] and that the up-regulation of the NK-1 receptor mRNA expression in chronic pancreatitis has a strong relationship with the pain syndrome that these patients experience[47]. Thus, overexpression of the NK-1 receptor could be involved in chronic pancreatitis-associated cancer. It has also been reported recently that the truncated NK-1 receptor is overexpressed in colonic epithelial cells from patients with colitis-associated cancer, whereas the full-length is not affected[42]. Thus, the overexpression of NK-1 receptors could be used as a diagnostic marker to identify patients at risk of neoplasms and may serve as a useful therapeutic target in the treatment of chronic

inflammation-associated cancer.

NANOMOLAR CONCENTRATIONS OF SP INDUCE PANCREATIC CANCER CELL PROLIFERATION AND THE MIGRATION OF TUMOR CELLS

SP acts as a mitogen in normal and tumor cells (*e.g.*, neuroblastoma, astrocytoma, melanoma, retinoblastoma, glioma, melanoma, larynx carcinoma, gastric and colon carcinoma, lymphoblastic leukemia) *via* the NK-1 receptor, since the growth inhibition of many human tumor cells after the administration of NK-1 receptor antagonists is partially reversed by the administration of SP[4,5,33-38,48]. Regarding pancreatic cancer cells, nanomolar SP concentrations elicit the proliferation of the pancreatic cancer CAPAN-1, PA-TU 8902, BxPC-3 and MIA PaCa-2 cell lines[13,14]. By contrast, the mitogenic action of SP on these cell lines could be partially reversed by using NK-1 receptor antagonists such as L-733.060, L-732.138 or the drug aprepitant[12-14]. Many data indicate that SP in a universal mitogen in NK-1 receptor-expressing tumor cells. The undecapeptide can be synthesized and secreted by tumor and non-tumor cells and SP can be released from nerve terminal, and/or it can be released into blood vessels[4,5]. Through these paths, the peptide can exert a mitogenic action on tumor cells. The regulation of local tumor activity through sensory nerves containing SP is relevant, since the undecapeptide could modulate the growth of tumor cells, exerting a direct interaction between the nervous system and the tumor cells. Thus, SP could induce mitogenesis *via* the following mechanisms: (1) autocrine (SP is secreted from tumor cells); (2) paracrine (SP exerts a mitogenic action in endothelial cells); (3) SP is released from nerve terminals; (4) SP reaches the whole body through the bloodstream; this is regulated by the limbic system; and (5) endocrine (SP is released from the tumor mass into the blood vessels)[3-5].

There are multiple cell signaling pathways regulated by SP. After the activation of the NK-1 receptor by SP, an increase in DNA synthesis has been reported in tumor cells, and it seems that *via* the NK-1 receptor the undecapeptide activates members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38MAPK[45] (Table 1). Once activated, ERK1/2 is translocated into the nucleus, inducing proliferation and protecting the cell from apoptosis[5,7]. In tumor cells, SP increases the phosphorylation and activity of Akt or protein kinase B, a serine-threonine protein kinase that becomes activated *via* phosphatidyl-3-kinase (PI3K); the activation of Akt suppresses apoptosis[49,50]. By contrast, NK-1 receptor antagonists inhibit the basal activity of Akt[51] (Table 1). After it has bound to the NK-1 receptor, other effects are also exerted by SP in tumor cells: it activates phospholipase D and enhances forskolin-stimulated cyclic AMP-production; SP induces the release of interleukins, taurine and

glutamate; it mobilizes intracellular calcium; it induces the formation of inositol phosphate; it stimulates glycogen breakdown; and it influences glutamate and K^+ transport[5,52-56]. The release of interleukins, taurine and glutamate by tumor cells induces an inflammatory process, increasing the levels of SP and hence increasing tumor cell proliferation. Moreover, it has been reported that after binding to the NK-1 receptor SP stimulates glycogen breakdown and increases the intracellular Ca^{2+} concentration in astrocytoma cells. Both effects occur in a concentration-dependent manner. These effects are completely blocked by the NK-1 receptor antagonist CP-96345[55]. In addition, one of the most prominent metabolic alterations in cancer cells is the increase in aerobic glycolysis and the dependency on the glycolytic pathway for adenosine triphosphate generation, known as the Warburg effect, because most cancer cells predominantly produce energy by means of a high rate of glycolysis followed by lactic acid fermentation[57]. Growing tumor cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin; this occurs even if oxygen is plentiful. Thus, after binding to the NK-1 receptors located in tumor cells, SP causes glycogen breakdown and the glucose obtained would be used by tumor cells to increase their metabolism[55]. This mechanism could partly explain the Warburg effect. By contrast, NK-1 receptor antagonists block glycogen breakdown in tumor cells[55], and hence can counteract the Warburg effect[3] (Table 1). This new approach to the NK-1 receptor is very interesting because until now the main goal has been the inhibition of the glycolytic enzymes. However, this strategy has not provided any practical results. In cancer treatment, a reduction in glucose formation by blocking the NK-1 receptor may be possible and indeed easier using NK-1 receptor antagonists. Accordingly, without glucose the Warburg effect is not possible in cancer cells.

The migration of tumor cells is a crucial requirement for the development of metastasis and the progression of cancer. At present, over 90% of cancer deaths are derived not from the primary tumor but from the development of metastases[58]. Thus, a major goal in the treatment of cancer should be to inhibit the development of metastases. In this sense, it is known that tumor cell migration is induced by classical neurotransmitters (dopamine, noradrenalin) and peptides (*e.g.*, SP) and that such migration is inhibited after the administration of D_2 receptor, adrenoceptor or NK-1 receptor antagonists[5,59]. It is also known that after binding to the NK-1 receptor SP induces a rapid change in cellular shape (including blebbing) and that membrane blebbing is important in cell movement, cell spreading, and cancer cell infiltration[60,61]. It has recently been reported that SP is involved in pancreatic cancer perineural invasion and that in pancreatic cancer cells SP induces cancer cell proliferation and invasion as well as the expression of matrix metalloproteinase (MMP)-2. SP also promotes neurite outgrowth and the migration of pancreatic cancer cell clusters to the dorsal root ganglia of newborns[14].

NK-1 RECEPTOR ANTAGONISTS INHIBIT PANCREATIC CELL PROLIFERATION IN A CONCENTRATION-DEPENDENT MANNER

At a certain concentration, these antagonists inhibit 100% of tumor cells. NK-1 receptors antagonists are a broad group of heterogeneous chemical compounds (Figure 1 and Table 1). There are two groups: peptide NK-1 receptor antagonists and non-peptide NK-1 receptor antagonists.

Peptide NK-1 receptor antagonists

Most of the work carried out on the design and preparation of antagonists of the NK-1 receptor has focused on the introduction of *D*-amino acids[18]. However, their affinity is several orders of magnitude lower than that of natural agonists, and the metabolic instability of peptide NK-1 receptor antagonists and their inability to gain access to the central nervous system through the blood-brain barrier limit their usefulness for *in vivo* studies. In addition, these substances generally have a number of drawbacks, such as poor potency and a lack of the ability to discriminate between tachykinin receptors, partial residual agonist activity, mast cell degranulating activity, and neurotoxicity after administration in the central nervous system[22]. Some of these peptide NK-1 antagonists are[18]: [D-Arg1, D-Trp 7,9, Leu11] SP (Spantide I). This antagonist is neurotoxic and a potent histamine releaser from mast cells; H-D-Lys (Nicotinoyl)-Pro-[3-(3-pyridyl)-Ala]-pro-D-Phe83,4-Cl2)-Asn-DTrp-Phe-D-Trp-Leu-Nle-NH2 (Spantide Ⅱ). This antagonist is devoid of neurotoxicity; [D-Arg1, D-Trp5, 7, 9, Leu11] SP. This antagonist has anticancer effects in a variety of *in vitro* and *in vivo* models (*e.g.*, pancreatic cancer)[11, 62-65]; (D-Arg1, D-Phe5, D-Trp7, 9, Leu11) SP; (D-Arg1, D-Pro2, D-Trp 7,9, Leu11) SP; [Arg6, D-Trp7,9, MePhe8] SP (6-11); [D-Pro2- Trp7, 9] SP; [D-Pro4, D-Trp7, 9, 10, Phe11] SP (4-11); p-HOPA-DTrp-Phe-DTrp-Leu-Leu-NH2: NY-3238; DMePhe-DTrp-Phe-DTrp-Leu(CH2NH)Leu-NH2: NY-3460.

Non-peptide NK-1 receptor antagonists

Since non-peptide NK-1 receptor antagonists became available[66-68], an increasing number of papers describing new non-peptide antagonists have been published[69]. Thus, steroids (WIN- 51708, *etc.*), perhydroisoindolones (RP-67580, RP-73467, RPR-100.893, *etc.*), benzylamino and benzylether quinuclidine (CP-96345, L-709.210, *etc.*), benzylamino piperidines (CP-99,994, GR-203040, GR-205.171, CP-122.721, *etc.*), benzylether piperidines (L-733.060, L-741.671, L-742.694, *etc.*) and tryptophan based (L-732.138, L-737.488, *etc.*) NK-1 receptor antagonists have been reported[22]. Investigation into non-peptide NK-1 receptor antagonists is a fast-developing field. Some of these peptide NK-1 antagonists have been used in clinical trials and found to be safe. Examples are the drug aprepitant (Figure 1) and its prodrug fosaprepitant, casopitant (GW-679769), vofopitant (GR-205171),

L-759.274, CP-122.721, Ezlopitant (CJ-11.974), Rolapitant, L-754.030, Serlopitant and CJ-11.974[70].

The binding sites for NK-1 receptor antagonists and SP are different[5]. SP is hydrophilic and binds to the extracellular ends of the transmembrane helices and especially to the extracellular loops of the receptor, whereas NK-1 receptor antagonists are lipophilic and bind more deeply between the transmembrane Ⅲ-Ⅶ domains (Figure 1). After binding to the NK-1 receptor, NK-1 receptor antagonists could block the functions of SP (Table 1). The pharmacologic effect is related to stereochemical features and is not linked to chemical composition. The action is concentration- and time-dependent manner. At higher concentrations, the beneficial effect in the host is summative. Thus, the pharmacologic effects of the NK-1 receptor antagonists are: anxiolytic, antidepressant, antiemetic, antimigraine, antialcohol addiction or neuroprotector effect in the central nervous system, and they also play a role in analgesic, antiinflammatory, and hepatoprotector processes, as well as in antivirus proliferation (Table 1). Regarding cancer, NK-1 receptor antagonists exert an antitumor action (inducing tumor cell death by apoptosis), and they have antiangiogenesis effects and inhibit the migration of tumor cells[3-5] (Table 1). Therefore, the NK-1 receptor antagonists could be considered a new generation of anticancer drugs[3-5,71].

In 1993, Merck initiated studies on NK-1 receptor antagonists based on both CP-96,345 and CP-99,994. L-733.060 (Figure 1) is one of the compounds developed from CP-99,994. It is a 3,5-bistrifluoromethyl benzylether piperidine[72]. The administration of the NK-1 receptor antagonist L-733.060 produces analgesia[73] and antidepressive effects[74,75]. The compound has been suggested for the treatment of anxiety and mood disorders[76] and in inflammatory liver disease, most likely owing to its ability to inhibit the effects of SP[77]. In addition, it has been reported that the NK-1 receptor antagonist L-733.060 acts as an antitumor agent in several human tumor cell lines[13,38,78-81]. In fact, this antitumor action has been reported against pancreatic cancer cell lines[13,14].

A morpholine nucleus that was introduced in the NK-1 receptor antagonist L-742.694 was found to enhance NK-1 receptor-binding affinity[82]. This nucleus was kept in further modifications of the molecule. In order to prevent possible metabolic deactivation, several refinements such as methylation on the C alpha of the benzyl ring and fluorination on the phenyl ring were introduced. These changes afforded the compound MK-869, which showed high affinity for the NK-1 receptor. MK-869 is also called aprepitant (Figure 1) and it has been tested for the treatment of several disorders. Those studies led the Food and Drug Administration to approve the drug Emend, which is indicated for chemotherapy-induced nausea and vomiting and is available for oral use[83]. A water-soluble phosphoryl prodrug for intravenous use, called fosaprepitant, is also available and is marketed as Ivemend[84]. It seems that aprepitant is effective for the treatment of depression[74,75], and it has recently been demonstrated that it is a broad-spectrum antitumor drug[12]. Moreover, the antitumor action of the drug aprepitant against pancreatic cancer cells has been reported. In fact, aprepitant inhibits 100% of pancreatic cancer cells in a concentration-dependent manner[12].

The NK-1 receptor antagonist L-732.138 (*N*-acetyl-*L*-tryptophan 3,5-bis (trifluoromethyl) benzyl ester) (Figure 1) shows a competitive and selective antagonism for the NK-1 receptor. It is approximately 1000-fold more potent in cloned human NK-1 receptors than in cloned human NK-2 and NK-3 receptors, and approximately 200-fold more potent in human NK-1 receptors than in rat NK-1 receptors[85]. The IC_{50} for the human NK-1 receptor expressed in Chinese Hamster Ovary cells is approximately 2.3 nmol[86]. It is known that the administration of L-732.138 produces an attenuation of hyperalgesia[87] and that L-732.138 is able to antagonize H(3) antagonist-induced skin vascular permeability. The antitumor action of the tryptophan-based antagonist L-732.138 against glioma, neuroblastoma and a larynx carcinoma cell lines has been also reported[80], as well as its antitumor action against pancreatic cancer cell lines[13,14].

The immunosuppressive cyclic undecapeptide cyclosporin A (CsA) is a naturally occurring fungal metabolite from *Tolypocladium inflatum Gams*. This molecule has been proposed to play a role in the treatment of human malignancies as an effective modifier of multidrug resistance. It is known that CsA has the pharmacological profile of an NK-1 receptor antagonist[88] and that CsA exerts an antitumor action due to its NK-1 receptor antagonist pharmacological profile in competition assay with SP. The antitumor action of CsA against pancreatic cancer cells occurs in a concentration-dependent manner and pancreatic tumor cells die by apoptosis[89]. However, in clinical practice this interesting therapeutic action of CsA is not possible because the high doses necessary to exert an antitumor action are associated with dangerous side effects, such as kidney failure.

Taking the above data together, it seems that the antitumor action of NK-1 receptor antagonists against pancreatic cancer cells would be due to stereochemical features and that it is not linked to the chemical composition of the antagonists[71] (Table 1), since different compounds (L-733.060, a piperidine derivative; aprepitant, a morpholine derivative; L-732.138, a tryptophane derivative ; CsA, a cyclic undecapeptide) exert an antitumor action (Figure 1). These compounds have only one thing in common: their affinity for the NK-1 receptor.

ANTITUMOR ACTION OF THE NK-1 RECEPTOR ANTAGONISTS IS MEDIATED THROUGH THE NK-1 RECEPTOR AND TUMOR CELLS DIE BY APOPTOSIS

As reported above, the NK-1 receptor antagonists (L-733.060, L-732.138, the drug aprepitant, *etc*.) exert an antitumor action[4,5,33-38] (Figure 1). In particular, these antagonists exert this action against human glioma, larynx

carcinoma, neuroblastoma, rhabdomyosarcoma, leukemia, astrocytoma, osteosarcoma, lymphoma, retinoblastoma, melanoma, lung, breast, and gastric, and colon carcinoma cell lines[4,5,33-38,90,91], as well as against pancreatic cancer cell lines[13,14]. The antitumor action of L-733.060 against human cancer cell lines is more potent than that of aprepitant, and the antitumor action of aprepitant is more potent than that of L-732.138[4,5]. NK-1 receptor antagonists block the SP-induced mitogen stimulation of tumor cells, and they inhibit tumor cell growth in a dose-dependent manner[4,5] (Table 1).

After binding to NK-1 receptors overexpressed in tumor cells, NK-1 receptor antagonists activate the apoptotic machinery and these cells (*e.g.*, pancreatic cancer, *etc.*) die by apoptosis[4,5,12,33,34,38]. Thus, the induction of apoptosis represents a highly suitable approach to cancer treatment, although currently little is known about the mechanisms responsible for the induction of apoptosis in tumor cells. Despite this, it has been reported that the blockade of NK-1 receptors by NK-1 receptor antagonists inhibits the basal kinase activity of Akt. Tumor cells develop strategies to neutralize the multiple pathways leading to cell death, and it has been suggested that one of the most important of these is the expression of the NK-1 receptor[92]. This strategy renders tumor cells highly dependent on the SP stimulus, which provides a potent mitotic signal. This signal could counteract the different death signal pathways activated in tumor cells. The absence of the mitotic signal when the receptor is blocked with NK-1 receptor antagonists could tilt the balance within the cell to favouring apoptotic/death signals, and hence the cell would die[92]. The data reported suggest that NK-1 receptor antagonists could inhibit a large number of tumor cell types in which NK-1 receptors are overexpressed[3-5,33,34,37], and that NK-1 receptor antagonists could be candidates for broad-spectrum antineoplastic drugs including pancreatic cancer[3-5,13,14]. In general, NK-1 receptor antagonists are safe, since the administration of NK-1 receptor antagonists does not induce serious side effects[5,72,93-96], although headaches, hiccupping, vertigo and drowsiness have been reported in humans after their administration[71,95,96] (Table 1). The safety of aprepitant against human fibroblasts has been also demonstrated: the IC$_{50}$ for fibroblasts is three times higher than the IC$_{50}$ for tumor cells[12]. Moreover, the IC$_{50}$ for non-tumor cells is 90 μmol/L but the IC$_{100}$ for tumor cells is 60 μmol/L approximately[12].

Furthermore, it has been reported that the use of chemotherapy and/or radiation therapy and NK-1 receptor antagonists affords a synergistic antitumor action and decreases the side effects of chemotherapy and radiation therapy[5,97,98] (Table 1). Furthermore, it has been suggested that the co-administration of NK-1 receptor antagonists and microtubule-destabilizing agents (*e.g.*, vinblastine) could be useful in cancer, since these compounds have a synergic effect[5,98] (Table 1). This combination is synergistic for the growth inhibition of NK-1 receptor-possessing cancer cells, but not for normal cells. A better

understanding of the mechanisms underlying this interaction is needed in order to assess the clinical relevance of this novel synergistic combination. Moreover, synergism has been reported for the combination of L-733.060 with common cytostatic drugs (adriamycin, mitomycin, ifosfamide, cisplatin) in MG-63 human osteosarcoma cells, but not in non-malignant HEK293 cells[99]. Pretreatment of HEK293 with L-733.060 prior to exposure to cytostatic drugs partially protected HEK293 cells from inhibition by these drugs[99].

NK-1 RECEPTOR ANTAGONISTS INHIBIT ANGIOGENESIS IN PANCREATIC CANCER XENOGRAFTS

Neovascularization or neoangiogenesis is a sequential process, with early endothelial proliferation followed by new vessel formation and increased blood flow, accompanied by maturation of endogenous neurovascular regulatory systems occurring late in this process in inflamed tissues[100]. The growth of new vessels from a pre-existing vasculature is a common feature of chronic inflammation (early neoangiogenesis is a key step in the transition from acute to persistent inflammation) and wound healing. Neoangiogenesis, a hallmark of tumor development, has also been associated with increased tissue innervation and the expression of NK-1 receptors. In a large majority of tumors investigated, SP and NK-1 receptors are found in the intra and peritumor blood vessels[6]. These findings have been reported specifically in pancreatic cancer[10]. SP, a main mediator of neurogenic inflammation through the release of the peptide from peripheral nerve terminals, is involved in the growth of capillary vessels *in vivo* and in the proliferation of cultured endothelial cells *in vitro*. Additionally, it is known that the proliferation of endothelial cells by NK-1 receptor agonists (SP or SP analog agonists) increases in a concentration-dependent manner (NK-1 receptor antagonists block the proliferative action of SP), whereas the action of selective NK-2 and NK-3 receptor agonists has no significant effects on the proliferation of endothelial cells. These findings indicate that NK-1 receptor agonists (*e.g.*, SP) can stimulate the process of neovascularization directly, probably through the induction of endothelial cell proliferation[101], and that SP enhanced angiogenesis results from a direct action on microvascular NK-1 receptors. Thus, through such receptors found at high density in blood vessels SP may strongly influence vascular structure and function inside and around tumors by increasing tumor blood flow and by fostering stromal development[6]. By contrast, it has been reported that NK-1 receptor antagonists inhibit endothelial cell proliferation and angiogenesis in a concentration-dependent manner[101] (Table 1). It has also been reported that the [D-Arg1, D-Trp5,7,9, Leu11] SP analog antagonist (SPA, broad-spectrum GPCR antagonist, peptide NK-1 receptor antagonist) has an antitumor action[11]. It is known

that in ductal pancreatic cancer cells expressing NK-1 receptors, NK-1 receptor antagonists induce the synthesis of proangiogenic chemokines and that in HPAF-II, a well-differentiated pancreatic cancer cell line, peptide NK-1 receptor antagonists inhibit Ca^{2+} mobilization and DNA synthesis[11]. These antagonists also significantly attenuated the growth of HPAF-II tumor xenografts in nude mice beyond the treatment period. Interestingly, one peptide NK-1 receptor antagonist (SPA, broad-spectrum GPCR antagonist) markedly increases apoptosis but moderately decreases the proliferation marker Ki-67 in tumor xenografts, implying additional mechanisms for the significant growth inhibitory effect[11]. HPAF-II cells express ELR$^+$ CXC chemokines, including interleukin-8/CXCL8, which bind to CXCR2 (a member of the GPCR superfamily) and promote angiogenesis in many types of cancer, including pancreatic cancer. A salient feature of these results is that peptide NK-1 receptor antagonists markedly reduced tumor-associated angiogenesis in HPAF-II xenografts *in vivo*. The data suggest that peptide NK-1 receptor antagonists (SPA, broad-spectrum GPCR antagonist) attenuate tumor growth in pancreatic cancer *via* a dual mechanism involving both antiproliferative and antiangiogenic properties[11]. Thus, the dual-inhibitory effect of peptide NK-1 receptor antagonists could be of significant therapeutic value in pancreatic cancer, when used in combination with other anticancer drugs. In sum, all these data indicate that the SP/NK-1 receptor system controls neoangiogenesis in pancreatic cancer and that, in addition, this system could also regulate the growth of the pancreatic tumoral mass, since NK-1 receptors are overexpressed in tumoral cells and in peritumoral pancreatic cancer tissues[10]. Thus, by using NK-1 receptor antagonists (peptide or non-peptide), the NK-1 receptor could be used as a target to inhibit both neoangiogenesis and the growth of pancreatic cancer (Figure 1 and Table 1).

Accordingly, targeted therapies for pancreatic cancer offer new ways to search for potentially more effective strategies. Thus, the use of NK-1 receptor antagonists in chronic pancreatitis could: (1) improve chronic inflammation; (2) improve pain; and (3) prevent the chronic pancreatitis associated with cancer. The use of NK-1 receptor antagonists in pancreatic cancer could exert: (1) an antitumor action, by inhibiting pancreatic cancer cell proliferation (tumor cells die by apoptosis); (2) antiangiogenic properties; and (3) inhibition of the migration of pancreatic cancer cells (preventing invasion, infiltration and metastasis). Thus, the antitumor action of NK-1 receptor antagonists in pancreatic cancer could be specifically for a single target: the NK-1 receptor (Figure 1). The mechanisms of action of NK-1 receptor antagonists are the opposite of those involved in classic chemotherapy. In addition, NK-1 receptor antagonists not only exert an antitumor action, but also elicit beneficial effects in the host such as anti-inflammatory, analgesic, anxiolytic, antidepressant, antiemetic, hepatoprotector and neuroprotector effects[4,5] (Table 1).

SAFETY OF NK-1 RECEPTOR ANTAGONISTS IN HUMAN CLINICAL TRIALS

As reported above, an upregulation of the SP/NK-1 receptor system occurs in human pancreatic cancer cells and hence the NK-1 receptor can be considered as an important target for the treatment of this disease. The overexpression of the NK-1 receptor in human pancreatic cancer cells suggests that the administration of NK-1 receptor antagonists is an excellent strategy for the treatment of this disease (these antagonists, after binding to NK-1 receptors, induce the apoptosis of tumor cells) and in addition fewer side effects should be expected after the administration of these drugs to patients, since NK-1 receptor antagonists are specific for a determined target, the NK-1 receptor, which is overexpressed in cancer cells and it is involved in the viability of tumor cells[3]. It should be noted that the IC100 for cancer cells is 60 μmol/L approximately but the IC50 for non-tumor cells is 90 μmol/L[12].

Many studies have reported the absence of serious side effects when non-peptide NK-1 receptor antagonists have been administered to humans[71]. It is known that the NK-1 receptor antagonist GR-205171 alleviated anxious symptoms in patients with social phobia[102]. Several non-peptide NK-1 receptor antagonists (*e.g.*, casopitant, orvepitant, vestipitant, vofopitant) have been also tested in human clinical trials for the treatment of depression, anxiety disorders, post-traumatic stress disorder, alcoholism, panic disorder and schizophrenia[103,104]. In some trials, these antagonists exerted an anxiolytic or an antidepressant action and in all the cases showed a low side effect profile. Moreover, the analgesic action of the NK-1 receptor antagonists aprepitant, lanepitant (LY-303870), AV-608 and CJ-11.974 has been tested in human trials and in all the cases the drug was ineffective in relieving pain (*e.g.*, neuropathic pain, visceral pain, osteoarthritis, fibromyalgia)[105]. However, the NK-1 receptor antagonist CP-99994 decreased postoperative dental pain[106]. NK-1 receptor antagonists have been also tested for the treatment of migraine. Thus, lanepitant was ineffective in migraine prevention and acute migraine; RPR-100.893 had no effects on migraine attacks; L-758.298 failed to abort migraine attacks, and GR-205171 was ineffective against the treatment of migraine[106]. Moreover, it has been reported that HIV-infected adults not receiving antiretroviral therapy, low (125 mg) and high (250 mg) doses of aprepitant (daily, for 14 d) were found to be safe[107]. Neurological adverse events (headache, hypersomnia, lightheadedness, dizziness) were observed in the 50% of the patients that received a higher dose of the NK-1 receptor antagonist, whereas insomnia was reported in those treated with 125 mg of aprepitant (11.1% patients). In both groups, the concentration of SP in plasma decreased. Gastrointestinal, ocular/visual, dermatological and systemic adverse events were also reported in the patients treated with aprepitant[107]. No changes in sleep

quality, anxious mood, depressed mood or neurocognitive measures were found[108].

Despite the large number of non-peptide NK-1 receptor antagonists reported, the only NK-1 receptor antagonist used currently in clinical practice is the drug aprepitant (Emend, MK-869, L-754.030) (oral) and its intravenously administered prodrug, fosaprepitant[3]. Fosaprepitant is rapidly converted to aprepitant *via* the action of ubiquitous phosphatases[108]. Both NK-1 receptor antagonists are used for the prevention of chemotherapy-induced nausea and vomiting and post-operative nausea and vomiting[70]. Many clinical human trials have reported the efficacy and safety of aprepitant/fosaprepitant for the treatment of emesis[70]. No serious adverse events were found. Aprepitant was well tolerated: no grade 3 or higher toxicities related to aprepitant were reported, whereas the adverse events mostly observed were fatigue, diarrhoea, febrile neutropenia, headache, dyspnea, constipation and hiccups[109].

Accordingly, novel possibilities for translational research are emerging for improving the treatment of diseases in which the SP/NK-1 receptor system is upregulated and hence, in particular, the use of NK-1 receptor antagonists in oncology therapy is quite promising according to the data obtained from preclinical studies[3]. Aprepitant is an excellent candidate for testing its antitumor, antimigratory and antiangiogenic action in human clinical trials since a large part of the required safety and characterization studies for aprepitant have already been carried out (aprepitant is already available in clinical practice for the treatment of emesis)[70]. Moreover, aprepitant has been developed as a nanoparticle formulation to enhance exposure and to minimize food effects. In humans, the nanoparticle formulation increased 3 times-4 times the bioavailability of this NK-1 receptor antagonist[110]. It has been also demonstrated in an *in vivo* study that fosaprepitant reduced significantly the tumor volume of MG-63 human osteosarcoma xenografts[99].

It seems that by increasing the number of days on which aprepitant is currently administered and using higher doses of aprepitant than those used in chemotherapy-induced nausea and vomiting this NK-1 receptor antagonist could be effective in cancer (*e.g.*, pancreatic cancer)[3]. However, these issues should be investigate in depth. By increasing the dose of aprepitant, higher and undescribed side effects may occur, although it has been reported that in patients with depression a dose of 300 mg/d of aprepitant was well tolerated and no significant difference in the frequency of adverse events was observed as compared with placebo[3].

CONCLUSION

The SP/NK-1 receptor system plays an important role in the development of pancreatic cancer, neoangiogenesis and metastasis. It seems that SP acts as a mitogen for pancreatic tumor cells overexpressing NK-1 receptors and that NK-1 receptor antagonists also induce apopto-sis in tumor cells. Research into the involvement of the SP/NK-1 receptor system in pancreatic cancer must continue in forthcoming years since it is necessary to explore new and effective therapeutic interventions in pancreatic cancer research. It is important to seek strategies targeting tumor-specific molecular derangements. This is the case of the NK-1 receptor, which is overexpressed in pancreatic tumor cells and tumor samples. NK-1 receptor antagonists induce the death of tumor cells by apoptosis. Accordingly, the NK-1 receptor is a promising target in the treatment of pancreatic cancer and NK-1 receptor antagonists could be considered as drugs for the treatment of this tumor. This conclusion is based on the following data: (1) after binding to the NK-1 receptor, SP induces pancreatic tumor cell proliferation, angiogenesis and the migration of pancreatic tumor cells (invasion, infiltration and metastasis); and (2) by contrast, NK-1 receptor antagonists inhibit pancreatic tumor cell proliferation (tumor cells die by apoptosis), have antiangiogenic properties in pancreatic cancer, and block the migratory activity of pancreatic tumor cells. Currently, in clinical practice there are few new drugs against the treatment of pancreatic cancer. However, it has been demonstrated *in vitro* and *in vivo* that NK-1 receptor antagonists exert an antitumor activity against pancreatic cancer cells. At the present, there are more than 300 NK-1 receptor antagonists[69] and this means that there are more than 300 potential drugs against the treatment of pancreatic cancer. Thus, it is crucial to test the antitumor action of NK-1 receptor antagonists in human clinical trials. In this sense, the antitumor action of NK-1 receptor antagonists already available in clinical practice for the treatment of emesis (*e.g.*, aprepitant) should be tested in clinical trials. It has previously been reported that the administration of aprepitant is well tolerated and is associated with minimal side effects. Indeed, at 300 mg/d of aprepitant was well tolerated and no significant difference in the frequency of adverse events were observed in comparison with placebo administration[71]. It is also known that, *in vitro*, aprepitant exerts an antitumor action against human pancreatic tumor cells[12]. In sum, all the data point to the notion that the NK-1 receptor could be a new and promising therapeutic target in pancreatic cancer and that NK-1 receptor antagonists could open the door to a new and promising generation of anticancer drugs against pancreatic cancer.

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Hedgehog signaling pathway as a new therapeutic target in pancreatic cancer

Hideya Onishi, Mitsuo Katano

Hideya Onishi, Mitsuo Katano, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
Author contributions: Onishi H and Katano M analyzed the data; Onishi H wrote the paper.
Correspondence to: Hideya Onishi, MD, PhD, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. ohnishi@surg1.med.kyushu-u.ac.jp
Telephone: +81-92-6426220 Fax: +81-92-6426221

Core tip: Hedgehog (Hh) signaling is involved in the induction of malignant potential in pancreatic cancer, controlling processes of proliferation, invasiveness and tumorigenesis. This phenotypic change is closely associated with the nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor, both in an autocrine and paracrine manner. Hh signaling is also capable of maintaining pancreatic cancer stem cells, and may be activated under conditions of tumor hypoxia. Thus, the Hh signaling pathway may represent a potential therapeutic target for patients with refractory pancreatic cancer and the use of Hh inhibitors will likely play an important role in future therapeutic strategies.

Abstract

Pancreatic cancer is one of the most aggressive and difficult cancers to treat. Despite numerous research efforts, limited success has been achieved in the therapeutic management of patients with this disease. In the current review, we focus on one component of morphogenesis signaling, Hedgehog (Hh), with the aim of developing novel, effective therapies for the treatment of pancreatic cancer. Hh signaling contributes to the induction of a malignant phenotype in pancreatic cancer and is responsible for maintaining pancreatic cancer stem cells. In addition, we propose a novel concept linking Hh signaling and tumor hypoxic conditions, and discuss the effects of Hh inhibitors in clinical trials. The Hh signaling pathway may represent a potential therapeutic target for patients with refractory pancreatic cancer.

Key words: Hedgehog signaling pathway; Pancreatic cancer; Cancer stem cells; Hypoxic condition; Therapeutic target

INTRODUCTION

Pancreatic cancer remains one of the deadliest cancers, with an overall survival rate of < 5%[1]. An underlying reason for this may be that few patients undergo curative, surgical operations because of the advanced stage of the cancer at the time of diagnosis. Furthermore, apart from chemotherapy and radiation therapy, there are no effective, alternative therapies for the treatment of refractory pancreatic cancer, and as such, the development of novel therapeutic strategies is urgently required. Recently, it was shown that the Hedgehog (Hh) signaling pathway, which plays a key role in morphogenesis signaling, is re-activated in pancreatic cancer[2]. Hh signaling contributes to tumor aggressiveness, affecting
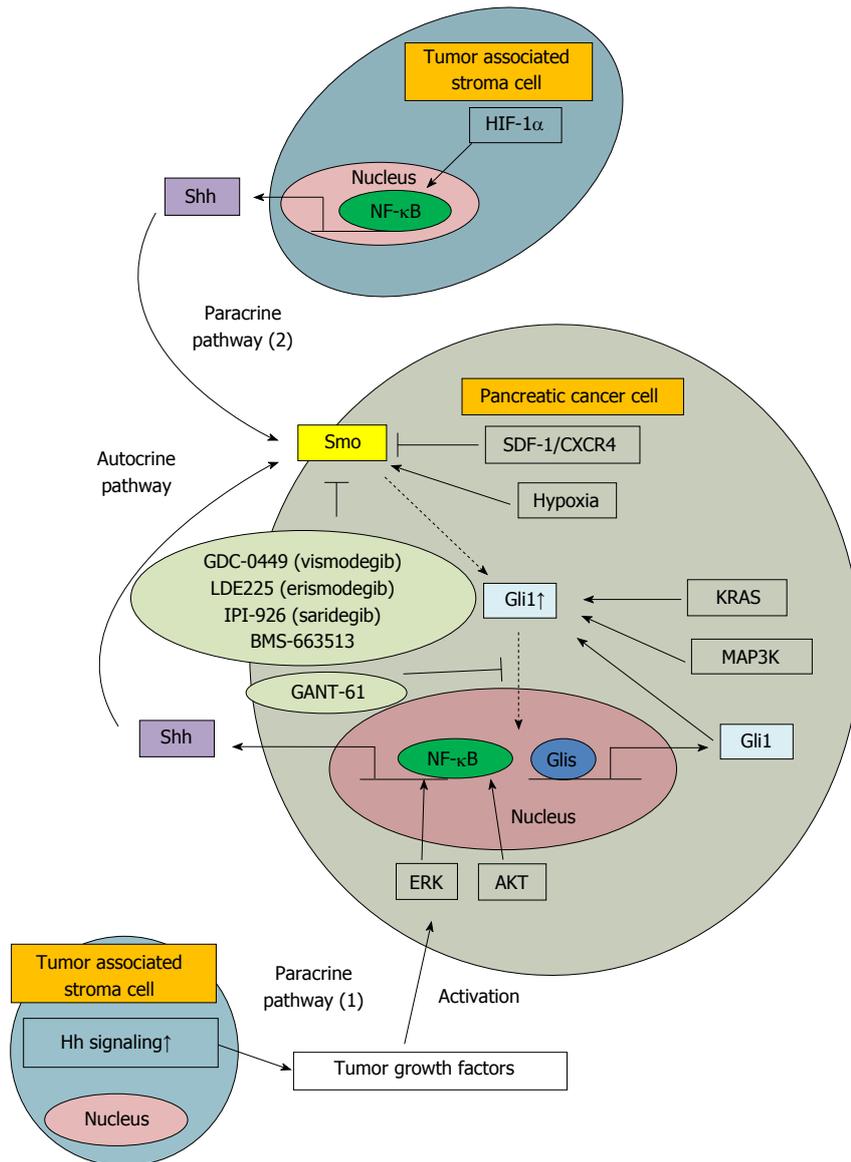
Figure 1 Schematic review. Hedgehog (Hh) signaling is activated in both autocrine and paracrine pathways. Tumor associated stroma cells play a pivotal role in tumor progression related to activation of Hh signaling [paracrine pathways (1) and (2)]. Induction of sonic Hh (Shh) is closely associated with activation of the nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor in pancreatic cancer. Shh is produced by NF-κB activation in pancreatic cancer cells and tumor associated stroma cells. Pathways contributing to Smo and Gli1 activation include SDF-1/CXCR4, hypoxia, KRAS and MAP3K. The effects of Hh inhibitors including GDC-0449 (vismodegib), LDE225 (erismodegib), IPI-926 (saridegib), BMS-663513 and GANT-61 in clinical trials are under investigation. Dotted arrows show components of the Hh signaling pathway in tumor cells focused on in this review.

key tumorigenic processes such as proliferation, invasion and progression of cancer cells. Therefore, inhibitors targeting Hh signaling have drawn significant attention as novel, molecularly targeted drugs. Hh signaling components including Patched and Smoothened (Smo) have been detected in almost 70% of human pancreatic cancer specimens and consequently, Hh signaling may play a critical role in the genesis of pancreatic cancer cells[2]. In this review, we summarize recent efforts in the development of new, therapeutic strategies to treat pancreatic cancer, targeting the Hh signaling pathway.

HH SIGNALING PATHWAY

The Hh signaling pathway plays a pivotal role in em-

bryonic patterning and growth control, acting as a morphogen, mitogen and inducing factor of developing organs[3-7]. Hh signaling normally ceases after embryogenesis, however in various cancers, including pancreatic cancer, Hh signaling is re-activated[8]. Therefore, the regulation of Hh signaling in pancreatic cancer likely plays important role in tumorigenesis. The Hh signaling pathway is composed of Hh proteins (sonic Hh; Shh, Indian Hh and Desert Hh), the 12-transmembrane Patched proteins (Patched 1 and Patched 2), the 7-transmembrane protein, Smo and the 5-zinc-finger transcription factors, Gli1, Gli2 and Gli3[9-11]. In the absence of Hh ligand, Patched suppresses Smo, which is the driving protein for Hh signaling, and Gli2 and Gli3 are cleaved by ubiquitin ligases to generate transcriptional repressor isoforms[12-14].

In contrast, in the presence of Hh ligand, inhibition of Smo by Patched is released, Smo is activated, and Gli2 and Gli3 are transmitted to the nucleus as full-length activators leading to the transcription of target genes such as Patched and Gli1[12-14]. Recent studies demonstrated the existence of primary cilia on the cell surface and showed that Smo moves from the cytoplasm to primary cilia in the process of activation[15]. One of the target genes of Hh signaling; Ptch and Gli1 regulate the transcription of the Hh responsive genes by themselves[16]. Other target genes of Hh signaling are the cell cycle regulator Cyclin D1, p21 and N-Myc which plays important role for carcinogenesis and is also typically dysregulated in the cancer cells[7,17,18]. The Hh signaling pathway is unique because several components of this pathway consist of both oncogenes and cancer suppressor genes.

HH SIGNALING AND THE INDUCTION OF MALIGNANT POTENTIAL IN PANCREATIC CANCER

Originally, the relationship between Hh signaling and tumorigenesis was reported following the association of mutations in genes such as *Gli1*, *Patch* and *Smo* in glioblastoma, basal cell carcinoma and rhabdomyosarcoma[19-21]. In pancreatic cancer, ligand-dependent activation of Hh signaling, but not genomic mutation, was first reported[2]. Previous studies have also shown that Shh overexpression is sufficient to initiate pancreatic intraepithelial neoplasia (PanIN)-like precursor lesions[2,22]. At present, this ligand-dependent pathway is thought to be the major mechanism underlying Hh signaling activation. Two distinct ligand-dependent activation pathways exist; autocrine and paracrine. In addition, association between chronic inflammation and the development of cancer has been recognized for several years[23-27]. In both autocrine and paracrine pathways, NF-κB plays a pivotal role. NF-κB is a transcription factor that controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion, adhesion, angiogenesis and apoptosis[28]. In the autocrine pathway, Shh is a direct transcriptional target of NF-κB, and proliferation of pancreatic cancer cells is accelerated *via* overexpression of Shh[29,30]. In the paracrine paradigm, tumor-associated stroma is important as a microenvironmental factor[31,32]. In one paracrine pathway, stroma cells surrounding pancreatic ductal adenocarcinoma cells, secrete tumor-growth factors through stromal Hh signaling activation[31]. This may explain why low concentrations of Hh signaling antagonist are sufficient to inhibit tumor growth [paracrine pathway (1), Figure 1][31]. In an alternative paracrine pathway, NF-κB-activated monocytes located in the tumor stromal area produce Shh, which stimulates the Hh signaling pathway in pancreatic cancer [paracrine pathway (2), Figure 1][33]. Inhibition of Hh signaling targets pancreatic stellate cells in the tumor-associated stroma, specifically reducing pancreatic tumor growth and metastasis[34,35]. In addition, Singh *et al*[36] showed that CXCL12/CXCR4 protein signaling induces Shh expression in pancreatic cancer *via* extracellular regulated kinase (ERK) and Akt kinase-mediated activation of NF-κB. Some other molecules affected by the activation of Hh signaling may also contribute to the induction of malignant potential in pancreatic cancer. Decrease in Cyclin D1by the inhibition of Hh signaling induces the G_0/G_1 arrest and inhibits cell proliferation[37]. Matrix metalloproteinase (MMP)-9 and MMP-2 locate the downstream of Gli1 and are involved with the invasiveness in pancreatic cancer[38,39].

HH SIGNALING AND PANCREATIC CANCER STEM CELLS

Solid tumor cancer stem cells were first identified in breast cancer as $CD24^{-/low}CD44^+$ cells[40]. $CD44^+CD24^+$ epithelial-specific antigen $(ESA)^+$ pancreatic cancer cells are reported to exhibit the stem cell characteristics of self-renewal and the ability to produce differentiated progeny[41]. Most importantly, cancer stem cells (CSCs) are characterized by features of resistance towards conventional chemotherapy and radiotherapy[42-45]. Pancreatic CSCs exhibit upregulation of Shh[46]. Recently, inhibition of Hh signaling was reported to inhibit the self-renewal of pancreatic CSCs and reverse chemoresistance[47]. Subsequent studies demonstrated that various agents were capable of inhibiting pancreatic CSCs *via* suppression of Hh signaling. For example, Tang *et al*[48] revealed that epigallocatechin-3-gallate, an active compound in green tea, inhibits the self-renewal capacity of pancreatic CSCs *via* inhibition of Hh signaling components including Smo, Ptch, Gli1 and Gli2. Other groups demonstrated that sulforaphane, a component of dietary cruciferous vegetables, decreases pancreatic CSC self-renewal *via* inhibition of Hh signaling components, Smo, Gli1 and Gli2[49,50]. Han *et al*[51] has revealed that suppression of Hh signaling by arsenic trioxide leads to the inhibition of the viability of pancreatic CSCs using animal models. A better understanding of the molecular pathways driving CSCs will lead to the development of effective, new therapeutic approaches for the treatment of pancreatic cancer.

As previously discussed, there are numerous reports describing $CD44^+CD24^+$ double positive cells in pancreatic CSCs. However to date, there have been relatively few studies investigating CD24 or CD44 molecules alone as therapeutic targets in pancreatic CSCs. CD24 is a unique molecule because it is described as a marker of pancreatic CSCs, whereas it is expressed at low levels or is absent in breast CSCs. CD24 is thought to act as an adhesion molecule[52,53]. Recently, truncated Gli1 was shown to induce clinically more aggressive cancer *via* the increased expression of CD24[54]. Ringel *et al*[55] showed that constitutive expression of CD44 variants may also be associated with the malignant state of invasive pancreatic carcinoma. However the precise roles CD24 and CD44 in pancreatic

CSCs remain unclear.

HH SIGNALING AND HYPOXIA

Pancreatic cancer is thought to occur under high levels of hypoxia[56]. Therefore, a detailed understanding of the hypoxic microenvironment is crucial for developing effective therapeutic approaches to treat this malignancy. Previous studies have shown that the oxygen concentration in venous blood and deep tumor environments is 5.3% and 1.3%, respectively[57,58]. Thus, to accurately analyze the molecular mechanisms underlying pancreatic cancer, experiments performed under hypoxic conditions are required. The relationship between hypoxia and Hh signaling activation was first reported in 2011, with a study showing that hypoxia activates Hh signaling pathway by upregulating Smo transcription[38]. Thereafter, it was reported that hypoxia induces epithelial to mesenchymal transition (EMT) *via* activation of Hh signaling[59]. Interestingly, under hypoxic conditions, activation of Hh signaling is independent of hypoxia inducible factor (HIF)-1α and is also ligand-independent, with no observable increase in Shh[38,59]. Conversely, Spivak-Kroizman *et al*[60] showed that hypoxia and desmoplasia led to more aggressive and therapy-resistant tumors *via* activation of Hh signaling by Shh, due to HIF-1α activation in the stroma. The mechanisms underlying activation of Hh signaling under hypoxic conditions remains unclear. However, given that Hh signaling is activated under tumor hypoxic conditions, this pathway may represent an important therapeutic target. Indeed, protein-bound polysaccharide decreases invasiveness and proliferation in pancreatic cancer by inhibition of Hh signaling, especially under hypoxia[39].

HH SIGNALING AND THERAPEUTIC APPROACHES IN PANCREATIC CANCER

Pancreatic cancer is often refractory to standard treatments, and many patients are unable to undergo surgery because of the advanced stage of disease at the time of diagnosis. Chemotherapy using gemcitabine and 5-FU derivatives, Tegafur-Gimeracil-Oteracil Potassium (S-1), are often used in Japan. However, combined use of Hh inhibitors with gemcitabine or 5-FU may induce chemoresistance[37]. One reason may be that gemcitabine and 5-FU are sensitive to S-phase and that Hh inhibitor often induces G1 arrest in cancer cells[37]. Conversely, several groups have shown that combined treatment with Hh inhibitors and gemcitabine has a synergistic effect on tumor growth in a xenograft model[61]. Combined use of Hh inhibitors and cisplatin, a cell cycle independent drug, may also have a synergistic effect[37]. Molecular targeting drug is now well established and the combined use of Hh inhibitors and other targeted drugs is currently being studied and utilized. For example, there is a possible synergistic relationship between Hh and epidermal growth factor receptor (EGFR) signaling pathways in pancreatic cancer[62-64]. Although combination therapy with Hh inhibitors remains controversial, these findings will be essential for developing new effective therapeutic strategies. Radiation is considered the third therapeutic strategy for the treatment of pancreatic cancer. Recently, focal radiation in combination with Hh inhibitors exhibited synergistic effects on reducing lymph node metastasis in pancreatic cancer[65]. Immunotherapy is anticipated as the fourth line of therapy after surgery, chemotherapy and radiation. In this approach, activated lymphocytes and dendritic cells (DCs) derived from patients with advanced cancer are often used. Recently, it was reported that Hh signaling is revitalized in activated lymphocytes and DCs derived from patients with advanced cancer and used for immunotherapy, and that this plays a pivotal role in the maintenance of their functions[66,67]. Therefore, Hh inhibitors may not have a synergistic effect when combined with immunotherapy.

Within the class of Hh inhibitors, recent drug development has focused on Smo inhibitors. Although exact patients' outcome has not been reported yet, Sekulic *et al*[68] has shown that the independently assessed response rate was 30% and 43%, and the median duration of response was 7.6 mo using two-cohort study with GDC-0449 (visnodegib) in metastatic and locally advanced basal-cell carcinoma. GDC-0449 and IPI-926 (saridegib) are currently under phase II clinical trials in metastatic, advanced and recurrent pancreatic cancer[69] and BMS-663513 is under phase I clinical trial[70]. A recent study demonstrated that LDE225 (erismodegib), a Smo antagonist, suppresses tumor growth and prolongs survival in a murine model of islet cell neoplasms[71]. Furthermore, GANT-61, a Gli transcription factor inhibitor, has been shown to inhibit pancreatic cancer stem cell growth[72]. An overview of Hh signaling inhibitors is shown in Figure 1. More recently, inhibition of Hh signaling has received significant attention as an anti-tumor strategy. Based on this, the relationship between Hh signaling and various materials has been reported. For instance, resveratrol, 3,4',5-trihydroxystilbene inhibits proliferation and induces apoptosis *via* Hh signaling in pancreatic cancer[73]. Curcumin, a phenolic compound extracted from Zigiberaceae turmeric, reverses EMT of pancreatic cancer by inhibiting Hh signaling[74]. Triparanol, a known cholesterol biosynthesis inhibitor blocking the 24-dehydrocholesterol reductase, suppresses pancreatic cancer tumor growth by deregulation of Hh signaling[75].

Gli1 is both a transcription factor and a target gene, as shown in previous reviews, and crosstalk between Hh signaling and other pathways has been demonstrated[8]. Gli1 is activated *via* several kinds of signaling pathways. In pancreatic cancer, various signaling pathways including KRAS[76], ERK[36], AKT[36], MAP3K[77] and SDF-1/CXCR4[78] are associated with Hh signaling (Figure 1). Because Gli1 is located downstream in many of these

pathways, it may represent a better therapeutic target.

CONCLUSION

In this review, we have summarized the development of pancreatic cancer treatment, with specific focus on the Hh signaling pathway. The Hh signaling pathway may represent an important therapeutic target in pancreatic cancer because this pathway is activated in the majority of pancreatic cancers and both ligand-dependent and independent inhibitors are effective. Hh inhibitor can successfully inhibit tumor growth and invasiveness *in vitro* and can be a promising drug, however, in clinical trial, it is not easy to verify the effectiveness of Hh signaling inhibitor. This reason may be that the actual function of Hh signaling molecules are not fully understood[79,80].

Hh signaling inhibitors should be effective in cancers in which Hh components are mutated such as basal cell carcinoma, basal cell nevus syndrome and medulloblastoma because Hh signaling is constitutively activated[81]. And in these cancers, Hh signaling inhibitors may become the first use drug in future clinical life. However, for other tumors, appropriate combination therapy may be required for the effective therapy. In January 2012, the Smo inhibitor, vismodegib, was clinically approved for the first time by the US Food and Drug Administration, for the treatment of unresectable or metastatic basal cell carcinomas of the skin[82]. Hh signaling inhibitors will now be used in pancreatic cancer as a monotherapy and in combination therapy with other chemodrugs, molecularly targeted drugs or radiation therapy.

ACKNOWLEDGMENTS

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World Clinical Pancreatology

2014 ADVANCES IN PANCREATIC CANCER

Minimally invasive radical pancreatectomy for left-sided pancreatic cancer: Current status and future perspectives

Chang Moo Kang, Sung Hwan Lee, Woo Jung Lee

Chang Moo Kang, Sung Hwan Lee, Woo Jung Lee, Department of Surgery, Yonsei University College of Medicine, Seoul 120-752, South Korea
Chang Moo Kang, Sung Hwan Lee, Woo Jung Lee, Pancreaticobiliary Cancer Clinic, Institute of Gastroenterology, Severance Hospital, Seoul 120-752, South Korea
Author contributions: All authors contributed to this work.
Correspondence to: Woo Jung Lee, MD, PhD, Department of Surgery, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, South Korea. wjlee@yuhs.ac
Telephone: +82-2-22282120 Fax: +82-2-3138289
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Abstract

Minimally invasive distal pancreatectomy with splenectomy has been regarded as a safe and effective treatment for benign and borderline malignant pancreatic lesions. However, its application for left-sided pancreatic cancer is still being debated. The clinical evidence for radical antegrade modular pancreatosplenectomy (RAMPS)-based minimally invasive approaches for left-sided pancreatic cancer was reviewed. Potential indications and surgical concepts for minimally invasive RAMPS were suggested. Despite the limited clinical evidence for minimally invasive distal pancreatectomy in left-sided pancreatic cancer, the currently available clinical evidence supports the use of laparoscopic distal pancreatectomy under oncologic principles in well-selected left sided pancreatic cancers. A pancreas-confined tumor with an intact fascia layer between the pancreas and left adrenal gland/kidney positioned more than 1 or 2 cm away from the celiac axis is thought to constitute a good condition for the use of margin-negative minimally invasive RAMPS. The use of minimally invasive (laparoscopic or robotic) anterior RAMPS is feasible and safe for margin-negative resection in well-selected left-sided pancreatic cancer. The oncologic feasibility of the procedure remains to be determined; however, the currently available interim results indicate that even oncologic outcomes will not be inferior to those of open radical distal pancreatosplenectomy.

Core tip: Minimally invasive (laparoscopic or robotic) radical distal pancreatosplenectomy is technically feasible and safe for margin-negative resection in well-selected left sided pancreatic cancer. Generally acceptable potential indications are proposed to include the following: (1) pancreas-confined tumors; (2) intact fascia layer between the distal pancreas and left adrenal gland/kidney; and (3) tumor 1-2 cm from celiac axis. The long-term oncologic feasibility remains to be discerned, but the currently available interim results are encouraging. Further clinical experience with this minimally invasive approach for left-sided pancreatic cancer should be accumulated by experienced surgeons. In the near future, surgical approaches should be specified according to the conditions of the individual pancreatic cancer case.

INTRODUCTION

With recent advancements in laparoscopic experience, techniques, and instruments, laparoscopic surgery has replaced conventional open surgery in most general surgical fields, even in cancer surgery. Despite the potential limitations of conventional laparoscopic surgery, many studies have prov-

en the oncologic feasibility and rationale for laparoscopic surgery in various malignant diseases, such as cancers of the esophagus[1,2], stomach[3,4], liver[5], colon[6,7], *etc*. However, it remains controversial whether minimally invasive surgery should be applied to treat pancreatic cancer.

Pancreatic cancer is known to be one of the most lethal gastrointestinal malignancies. As a monotherapy, margin-negative pancreatectomy can provide the essential clinical conditions for cure, but the resection rate is very low due to the advanced cancer stages that are usually present at the initial diagnosis. In addition, surgical techniques for margin-negative radical pancreatectomy are very difficult and complex procedures, even in the conventional open approach. Therefore, many surgeons greatly fear that the risk of incomplete surgery might arise when applying minimally invasive techniques to treat pancreatic cancers. Moreover, the lack of more advanced laparoscopic techniques and the limited amount of clinical evidence are some of the biggest obstacles to the use of laparoscopic approaches in the treatment of pancreatic cancer.

Still, several currently available studies have suggested that patients with pancreatic cancer may have appropriate backgrounds for the use of a minimally invasive approach to treat well-selected left-sided pancreatic cancers. First, unlike laparoscopic pancreaticoduodenectomy, laparoscopic distal pancreatectomy is generally regarded as a safe and effective treatment modality in benign and borderline malignant diseases[8]. Second, even laparoscopic subtotal (or extended) distal pancreatectomy can be feasible and safe[9]. Third, many laparoscopic gastric surgeons have already proven the oncologic safety and feasibility of laparoscopic perigastric lymph node dissection in the treatment of gastric cancer[10]. Fourth, the concept of radical antegrade modular pancreatosplenectomy (RAMPS)[11] is thought to be a reasonable approach for margin-negative and systemic lymph node clearance in left-sided pancreatic cancer. Fifth, the early detection of small and asymptomatic pancreatic cancer is expected to increase in the near future due to frequent routine medical check-ups. Finally, even though the data remain limited, a few encouraging studies have been published on the feasibility of a minimally invasive approach to pancreatic cancer[12-14].

Various types of minimally invasive pancreatectomy are currently feasible; however, in this review, we will address distal pancreatosplenectomy in the treatment of pancreatic cancer because this surgical procedure is popular and generally regarded as safe. Therefore, it is thought that laparoscopic distal pancreatectomy with splenectomy could be the initial step for generalizing the concept of a minimally invasive approach to well-selected pancreatic cancers.

CONCEPT OF RAMPS AS A MINIMALLY INVASIVE (LAPAROSCOPIC OR ROBOTIC) APPROACH

Strasberg *et al*[11,15] presented this modified distal pancreatosplenectomy technique in pancreatic cancer. In this meth-od, dissection proceeds from right to left after an early division of the pancreatic neck on one of the two posterior dissection planes to achieve negative posterior resection margins. The plane of dissection runs posteriorly in the sagittal plane along the superior mesenteric artery and celiac artery to the level of the aorta and then laterally, either anterior or posterior to the adrenal gland, for tangential margin clearance. The accompanying N1 lymph node dissection is based on the established anatomy of lymphatic drainage of the pancreas. The posterior dissection plane can be actively placed for tangential margin clearance. According to the posterior dissection plane of the pancreas, three types of RAMPS can be generally classified (Figure 1). Compared to the usual conventional technique for distal pancreatosplenectomy (dissection from left to right first and vascular control later[16]), RAMPS is thought to be more in line with general oncologic concepts, such as early vascular control and no-touch isolation with en bloc surgical resection. Therefore, when applying minimally invasive approaches to left-sided pancreatic cancer, the principles behind RAMPS should be incorporated, although the generally acceptable extent to which minimally invasive RMAPS can be applied must be determined first.

DETERMINING THE EXTENT OF MINIMALLY INVASIVE RAMPS AND POTENTIAL INDICATIONS

According to our surgical experiences with left-side pancreatic cancer, bloodless and margin-negative resection is an important factor in treating left-sided pancreatic cancer[14]; other reports have also supported this finding[17,18]. However, the use of combined adjacent organ resection has been associated with large amounts of intraoperative bleeding, transfusion, morbidity, and increased risks of a positive resection margin[19,20].

When correlating between the RAMPS surgical mode and the potential tumor behavior, several relationships can be identified (Figure 2, solid line). For example, in the case where posterior RAMPS 2 is selected for margin-negative resection, as opposed to anterior RAMPS, there is a high probability of a large tumor size, combined resection of adjacent organs, large amounts of intraoperative bleeding, and perioperative transfusions, as well as technically demanding, more aggressive tumor behaviors, such as actual margin positivity, peritoneal seeding, or hidden distant metastasis. In contrast, when considering the current technical feasibility of minimally invasive distal pancreatosplenectomy for bloodless and margin-negative resections, minimally invasive anterior RAMPS is well accepted; however, it would be very technically difficult to obtain margin-negative and bloodless resections in the case of minimally invasive posterior RAMPS 1 or RAMPS 2 (Figure 2, dotted line). Certainly, minimally invasive posterior RAMPS 1 and RAMPS 2 are also feasible [Figure 2, areas (B) and (C)], but it is thought that only a few expert laparoscopic surgeons can be fully responsible for those demanding surgical procedures[21]. Therefore, it is generally recommended that open aggressive pancreatectomy only

Figure 1 Mode of radical antegrade modular pancreatosplenectomy. A: Anterior radical antegrade modular pancreatosplenectomy (RAMPS); B: Posterior RAMPS 1; C: Posterior RAMPS 2. Dissection plane (yellow line) should be changed for clear tangential margin according to tumor condition (red circle).

Figure 2 Determining the extent of minimally invasive radical antegrade modular pancreatosplenectomy. The dotted line shows the technical feasibility of bloodless and margin-negative radical antegrade modular pancreatosplenectomy (RAMPS) by a minimally invasive approach, and the solid line represents the biological aggressiveness of tumors, according to the appropriate mode of RAMPS for margin-negative resection. Tentatively, minimally invasive anterior RAMPS is thought to represent a generally acceptable surgical extent for bloodless and margin-negative resections. Oncologically safe posterior RAMPS 1 and 2 might be difficult to perform using a minimally invasive approach. Note the marginal zone of (B). Only a few expert laparoscopic surgeons can be fully responsible for this region. Future directions include widening the area of (B) by means of technical evolution (shifting of the dotted line to the left) and improving early tumor detection (attenuating the slope of solid line). MIS: Minimally invasive surgery.
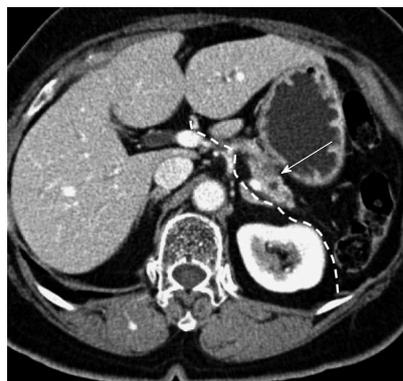
Figure 3 Potential indication for minimally invasive anterior radical antegrade modular pancreatosplenectomy. A 76-year-old female. A relatively pancreas-confined low density mass lesion is noted (arrow). The dotted white line indicates the dissection plane for minimally invasive anterior radical antegrade modular pancreatosplenectomy (RAMPS). The intact fascia layer between the pancreas and left adrenal gland/kidney can facilitate posterior margin clearance when removing the surgical specimen. The tumor is separated from the origin of the splenic artery, necessary for safe vascular control by introducing a minimally invasive technique. The patient underwent laparoscopic anterior RAMPS and has been followed for more than 1 year without evidence of tumor recurrence.

be performed for patients requiring posterior RAMPS 1 and 2. Consequently, when generalizing the concept of minimally invasive approaches to left-sided pancreatic cancer, it would be wise to limit the procedure to anterior RMAPS alone [Figure 2, area (A)][22]. This surgical extent will cover following potential tumor conditions: (1) pancreas-confined tumors; (2) intact fascia layer between the distal pancreas and left adrenal gland/kidney; and (3) tumor 1-2 cm from celiac axis (Figures 3 and 4).

CURRENT CLINICAL PRACTICE OF THE MINIMALLY INVASIVE APPROACH TO LEFT-SIDED PANCREATIC CANCER

Primitive evidence

Until now, many studies have proven the clinical benefit of laparoscopic distal pancreatectomy, with or without splenectomy, in benign and borderline malignant pancreatic disease. However, only a few previous studies have reported the laparoscopic approach for left-sided pancreatic cancer with available long-term survival outcomes[12,23-27]. Since Gagner *et al*[28] first reported laparoscopic distal pancreatectomy, with the advance of laparoscopic techniques and experiences, several other studies have been published, showing the technical feasibility,
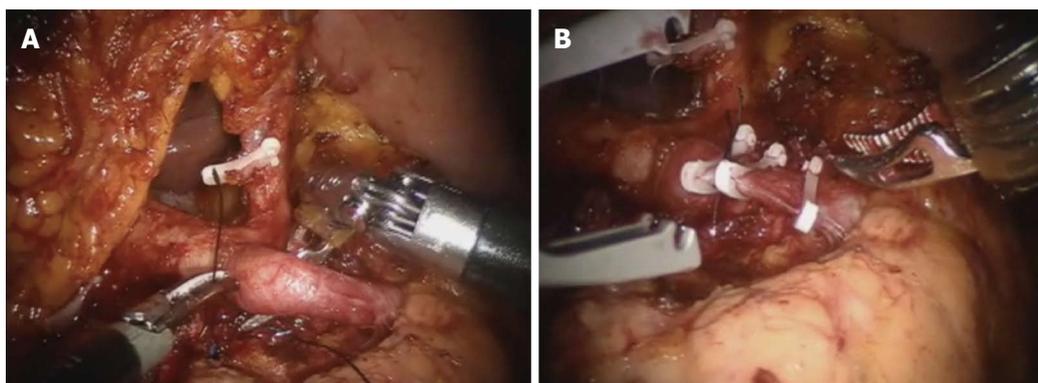
Figure 4 Adequate distance between celiac axis and tumor. Robotic anterior radical antegrade modular pancreatosplenectomy. The origin of the splenic artery is isolated (A) and ligated (B) by the robotic surgical system. For technically and oncologically safe minimally invasive vascular control, some cancer-free space is extremely necessary.

safety, and clinical benefit of laparoscopic distal pancreatectomy over open distal pancreatectomy. However, most reported cases of pancreatic cancer (ductal adenocarcinoma) treated by laparoscopic distal pancreatectomy were incidentally included in those series. As a result, we cannot fully assess the surgical quality based on relevant oncologic concepts. In addition, the lack of information on tumor characteristics, such as pT stage, pN stage, number of retrieved lymph nodes, margin status, and survival outcomes, creates difficulties in determining the oncologic feasibility of the laparoscopic approach to the left-sided pancreatic cancer[24-27,29,30]. For example, in one collective review performed in 2009[31], a final diagnosis of pancreatic ductal adenocarcinoma was found in 51 patients (9.8%, 51 out of 588 patients). However, the margin status was available in only 20 patients (39%). In addition, the number of retrieved lymph nodes in patients with pancreatic cancer was reported in only three articles[26,32,33] (12.5%, 3 of 24 articles identified). Not surprisingly, there is still a lack of long-term survival outcomes. Despite the efforts of several surgeons to perform laparoscopic distal pancreatectomy for pancreatic cancer, it was found that there is a substantial lack of evidence on the oncologic outcomes and surgical quality. Consequently, for the last several decades, we were uncertain whether the minimally invasive approach to left-sided pancreatic cancer was appropriate.

Intermediate evidence

Recently, several studies have been published that focused on the question of whether laparoscopic distal pancreatectomy is oncologically feasible.

DiNorcia *et al*[34] reported their experiences with laparoscopic distal pancreatectomy between 1991 and 2009. Seventy-one patients underwent laparoscopic distal pancreatectomy, and only 9 patients (12.7%) were reported to have malignant pathologies, including 3 cases of pancreatic ductal adenocarcinoma. Long-term survival outcomes were not analyzed; however, the margin-negative resection rate (2.8% *vs* 13%, $P < 0.01$) and mean number of retrieved lymph nodes [6 (range: 2.5-12.0) *vs* 8 (range:

3.0-13.0), $P = 0.29$] were shown to be comparable with those in a conventional open approach.

A recent multicenter analysis reported by Kooby *et al*[13] has provided the most encouraging and impressive evidence, considering the lack of long-term oncologic evidence of laparoscopic approaches to left-sided pancreatic cancer. This study showed that laparoscopic distal pancreatectomy is able to provide similar short- and long-term oncologic outcomes to those obtained with open distal pancreatectomy and suggested that laparoscopic distal pancreatectomy is an acceptable approach for the resection of the left-sided pancreatic cancer in selected patients. In the matched analysis of the overall survival for the patients undergoing an open ($n = 70$) versus a laparoscopic distal pancreatectomy ($n = 23$) for pancreatic cancer, the median survival was comparable among the two the groups (median 16 mo, $P = 0.71$).

In addition, Kim *et al*[35] also published the long-term outcomes of patients who were postoperatively diagnosed with malignancies after laparoscopic distal pancreatectomy. Of the 88 patients who underwent a laparoscopic distal pancreatectomy, 11 (12.5%) were subsequently diagnosed with malignancies in their postoperative pathologic reports. Pancreatic ductal adenocarcinoma was the most common (5 out of 11 patients), followed by invasive intraductal papillary mucinous neoplasm ($n = 3$), neuroendocrine carcinoma ($n = 1$), and so forth. During the follow-up period (range, 3-60 mo), they reported only 1 patient who died of cancer; all others were still alive. Thus, the authors carefully concluded that the postoperative outcomes among patients who were diagnosed postoperatively with malignant pancreatic disease are acceptable.

Although these retrospective studies were not able to suggest either standardized surgical procedures or proper indications, they did suggest potential oncologic outcomes and verify the technical feasibility of the laparoscopic approach to left-sided pancreatic cancer.

Recent advance evidence

More encouraging clinical data with intent-to-treat for

Table 1 Recent studies on minimally invasive radical antegrade modular pancreatosplenectomy and open distal pancreatosplenectomy in left-sided pancreatic cancer n (%)

Characterisc	Minimally invasive radical antegrade modular pancreatosplenectomy						Open distal pancreatosplenectomy			
	Fernández-Cruz et al[12]	Song et al[37]	Choi et al[36,38]	Magge et al[40]	Marangos et al[41]	Kanda et al[48]	Kooby et al[23]	Kang et al[14]	Mitchem et al[22]	Okada et al[50]
Surgical technique	RAMPS-based	RAMPS-based	RAMPS-based	RAMPS-based	Conventional technique-based	NA	NA	Conventional technique-based	RAMPS-based	Standard DP
Indication	Anatomic dissection No combined resection	No distant metastasis Not locally advanced	Relatively pancreas-confined, intact fascia layer, apart from celiac axis	Allegedly the same as open surgery, even when allowing adjacent organ combined resection	Apparently the same as open surgery, even when allowing adjacent organ combined resection	Resectable, no distant metastasis, no peritoneal seeding, no major vascular invasion	NA	Resectable, no distal metastasis, no peritoneal seeding, no major vascular invasion	Resectable, no distant metastasis, no peritoneal seeding	Resectable, not invading major vessel
Patients (n)	10	24	9	28	21	51	70	27	47	36
Age (yr)	NA	NA	64.8 (54-76)	67 (60-75)	63.1 (49-83)	62.7 (38-79)	65.9 ± 11.1	60.5 (47-75)	64.5 ± 10.3	68
Gender (male/female)	NA	16/8	5/4	9/19	6/15	34/17	27/43	20/7	20/27	23/13
Tumor size (cm)	NA	2.6 (1.4-10)	2.5 (1.2-6)	3.0 (2.2-3.6)	4.8 (1.0-10)	7 (< 2 cm) 44 (> 2 cm)	3.5 ± 1.4	3.9 (2.5-5.3)	4.4 ± 2.1	NA
pT stage (T1/T2/T3/T4)	NA	NA	0/0/9/0	NA	NA	2/5/20/24/0[1]	NA	1/4/20/2	1/4/41/1	1/2/15/17/0/1[1]
Retrieved LNs	14.5 (6-20)	10.3 ± 8.6	10.8 (2-23)	11 (8-20)	7.4 (0-26)	NA	12.3 ± 8.3	11.3 (5-18)	18.0 ± 11.7	NA
pN stage (N0/N1)	5/5	NA	2/7	12/16	14/9	25/26	NA	15/12	26/21	19/17
R0(%)/R1/R2	9 (90)/1/0	22 (91.7)/2/0	9 (100)/0/0	24 (86)/4 (14)/0	19 (90.5)/2/0	38 (74.5)/13	46/24	3/4/2020	81%/19%/0%	29 (80.5%)/6/1
Operation time (min)	320 (280-330)	225 (95-360)	368.3 (180-700)	260 (220-340)	NA	NA	216 ± 69	274 (97-453)	243.6 ± 93.5	203 (128-276)
Blood loss	720 (300-1300)	NA	360.1 (trace-1350)	200 (150-300)	NA	NA	751 ± 853	643.3 (100-1200)	744.3 ± 570.4	700 (10-2850)
Hospital stay (d)	8 (7-11)	9.5 (5-22)	9 (4-16)	6 (4-6)	5	NA	9.4 ± 4.7	21.2 (7-24)	11.3 ± 6.8	NA
Adjuvant chemotherapy	10 (100)	17 (70.8)	9 (100)	17 (100)	NA	NA	45 (64)	NA	NA	NA
Follow-up period (mo)	NA	9.95 (1.3-48.5)	20.7 (5.1-45.9)	NA	21.1 (0.5-108)	17.1	10	NA	26.4	25
Oncologic outcome	Median survival 14 mo	2-yr overall survival, 85.2%	2-yr disease free survival, 83.3%	NA	Median survival 19 mo	1, 3, 5-yr overall survival rate, 57.2%, 12.4%, 6.2%	Overall survival 16 mo	Median survival 27.9 mo, 5-yr survival, 28.9%	Median survival 25.9 mo, 5-yr survival, 30.4%	Median survival 32 mo, 2-yr survival 52%

Data are confined to pancreatic ductal adenocarcinoma alone. [1]UICC Cancer Staging System. NA: Not available; RAMPS: Radical antegrade modular pancreatosplenectomy.

minimally invasive approach to left-sided pancreatic cancer has been published. When considering RAMPS-based laparoscopic approaches to treating left-sided pancreatic cancer, only 5 studies investigated the interim or long-term follow up results on the safety and feasibility of minimally invasive distal pancreatectomy for pancreatic cancer (Table 1). A group in Barcelona[12] reported that the median survival of pancreatic cancers treated by laparoscopic RAMPS was 14 mo, comparable to the usual pancreatic cancers that were treated with an open surgery. This report is thought to be first to reveal the technical and oncologic feasibility of laparoscopic RAMPS in left-sided pancreatic cancer. In addition, despite the limited follow-up period, current interim results[36-38] strongly suggest that the possible oncologic outcomes following minimally invasive RAMPS will not be inferior to those of conventional open surgery in well-selected left-sided pancreatic cancer.

A group from Pittsburgh recently published their institutional historical experiences of minimally invasive (laparoscopic and robotic) distal pancreatectomy[39]. A total of 27 patients were reported to have undergone minimally invasive distal pancreatectomy for the treatment of pancreatic ductal adenocarcinoma. When excluding conversion cases

during laparoscopic distal pancreatectomy, the margin-positive resection rate was reported to be 4%, and the capacity for lymph node retrieval was up to 17 (range 10-19); these results are comparable with those of robotic distal pancreatectomy [R1 resection rate, 0% and nodal harvested, median 19 (range 17-27)], suggesting an acceptable quality of surgery in treating pancreatic cancer. They also analyzed retrospective 62 consecutive patients undergoing open distal pancreatectomy (ODP = 34) and minimally invasive distal pancreatectomy (MIDP = 28 with 5 conversions) for pancreatic ductal adenocarcinoma[40]. It was shown that overall survival after ODP or intended MIDP was similar after adjusting for comorbidity and year of surgery [relative hazard, 1.11 (95%CI: 0.47-2.62)]. These two studies still lack long-term oncologic outcomes (median follow up of 21 mo), however, no evidence was detected that MIDP was inferior to ODP in treating pancreatic cancer.

On the other hand, Marangos *et al*[41] published an interesting paper about their surgical experiences with laparoscopic distal pancreatosplenectomy for pancreatic exocrine carcinoma. Since 1997, they reported removing all lesions in the body and tail of the pancreas laparoscopically, and 29 patients with pancreatic cancer (11.6%, 29 out of 250 patients) underwent laparoscopic distal pancreatosplenectomy. Their approach was not based on RAMPS but rather on the conventional left-to-right technique. In addition, they did not perform formal lymph node dissection; instead, they only removed the enlarged or suspicious regional lymph nodes. The dissection plane and resection margins were carefully guided by laparoscopic intraoperative ultrasound. They reported an overall 93% R0 resection rate with a median survival of 23 mo (in particular, 19 mo for 21 pancreatic ductal adenocarcinomas), which is also comparable to the best open series[15,42]. It was noted that the median number of retrieved lymph nodes was smaller (5 nodes), but this did not translate into poor oncologic outcomes, again reminding us of the outcomes of previous prospective randomized controlled studies on standard and extended pancreaticoduodenectomy in the treatment of pancreatic head cancer[43-45]. In addition, in comparison with the oncologic outcomes from open radical surgery, perioperative and oncologic outcomes appear to be comparable between the minimally invasive radical distal pancreatectomy and the open approach (Table 1). One of the most significant weak points of the minimally invasive approach to pancreatic cancer is that the oncologic outcomes are still based on a short-term follow-up period, compared to that of open radical pancreatectomy[14,15,46-48]. However, recently, the single-center-based Pittsburgh group[40] reported a comparative analysis, including long-term survival, of 34 patients with open radical pancreatectomy and 34 with minimally invasive distal pancreatectomy in pancreatic ductal adenocarcinoma to determine the oncological safety and efficacy of minimally invasive surgery. They demonstrated no significant difference between two groups in tumor size (3.0 cm *vs* 3.0 cm), radiologic stage (I A/ I B/ II A/ II B, 3/12/10/6

vs 3/11/5/4), margin-negative resection (88% *vs* 86%), power of lymph node retrieval (12 *vs* 11), or lymph node metastasis (38% *vs* 57%) and similar postoperative complications, leading to equivalent survival in propensity score-adjusted overall survival analysis [relative hazard, 1.11 (95%CI: 0.47-2.62), *P* = 0.80]. Along with the multicenter case-matched analysis by Kooby *et al*[13], this study provides powerful evidence to support the technical feasibility of minimally invasive radical oncologic surgery. The study further shows that the quality of surgical specimens is quite acceptable and provides encouraging oncologic survival outcomes.

CHALLENGING ISSUES

Combined and vascular resection

Distal pancreatectomy with *en bloc* celiac axis resection (DPCAR) has been introduced for locally advanced left-sided pancreatic cancer involving the common hepatic artery and/or celiac axis, with perineural invasion in the nerve plexus surrounding these arteries[49,50]. In particular, Okada *et al*[50] recently concluded that DP-CAR is feasible and should be reserved for patients without tumors infiltrating either the portal venous or arterial system. Considering these circumstances, DP-CAR is suggested to be a safe procedure, similar to standard distal pancreatectomy in well-selected patients. Recent technological innovations and extensive surgical experiences are expanding the clinical applications for laparoscopic distal pancreatectomy. As a result, the technical feasibility of minimally invasive distal pancreatectomy with combined celiac trunk or portal vein resection has also been reported. Cho *et al*[21] reported the technical feasibility of pure laparoscopic DP-CAR, finding it safe and feasible to achieve R0 resections in selected patients with locally advanced pancreatic cancer. Giulianotti *et al*[51] and Boggi *et al*[52] also reported robotic pancreatectomy with vascular resection for locally advanced pancreatic tumors. In addition, Kendrick *et al*[53] reported 11 patients who underwent total laparoscopic pancreaticoduodenectomy with major venous vascular resection, including laparoscopic end-to-end vascular reconstruction, patch, and renal vein graft. However, patients with left-sided pancreatic cancer invading isolated superior mesenteric vein-splenic vein-portal vein confluence are rare, as most cases of pancreatic cancer are usually associated with celiac axis and superior mesenteric artery invasion[54], which will be determined as locally invasive pancreatic cancer (unresectable). In general, pancreatic surgeons must consider possible combined vascular resection in their surgical approaches to pancreatic cancer and should be prepared to meet this surgical demand. However, how many surgeons can be responsible for this advanced laparoscopic technique? How should the educational system be modified to reproduce this surgical skill?

Is only RAMPS the ideal approach?

The surgical approach of RAMPS has demonstrated favorable oncologic outcomes in treating left-sided pancreatic cancer[11,15,22,55]. The basic concept of RAMPS is,

of course, oncologically sound and reasonable; however, it is notable that no randomized controlled studies have tested the oncologic superiority between RAMPS and conventional radical distal pancreatectomy. There are several comparable reports showing similar survival outcomes to RAMPS[14,41,56]. An RCT should be performed to test whether the RAMPS procedure is superior to standard distal pancreatectomy. However, it is very difficult to organize a successful trial. Mitchem *et al*[22] have already commented on this issue, as follows: "However, the disparity between the number of cases available for study and the number required for a randomized trial makes this goal unattainable".

FUTURE PERSPECTIVES ON MINIMALLY INVASIVE LEFT-SIDED RADICAL PANCREATECTOMY

As shown in other gastrointestinal cancer surgeries, there has been an increasing clinical effort to apply the laparoscopic approach to left-sided pancreatic cancer. However, procedural standardization and surgical indications have not yet been established. Currently, RAMPS seems to be a reasonable approach, with encouraging oncologic outcomes in the treatment of left-sided pancreatic cancer[22]. Nevertheless, it might be difficult to expand the use of minimally invasive RAMPS to all left-sided pancreatic cancers because these cancers are usually found in advanced cancer stages. However, the clinical conditions required to widen the area (B) in Figure 2, such as technical evolution (right sided-shift of dotted line in Figure 2) and the early detection of the cancer (attenuating slope of the solid line), would facilitate the clinical application of minimally invasive RAMPS in well-selected cases of left-sided pancreatic cancer.

Recently, the use of radical pancreatectomy followed by neoadjuvant chemoradiation therapy has been successfully applied for the treatment of advanced pancreatic cancers[57-59]. In considering the future circumstances of potent chemoradiation therapy for the treatment of pancreatic cancers, minimally invasive RAMPS following neoadjuvant chemoradiation therapy would be another potential option for well-selected patients. In particular, considering the technical advances of combined vascular resection in treating pancreatic cancer, the indications for minimally invasive radical distal pancreatectomy should be expanded in the near future. In addition, many academic institutions seem to be carefully accumulating clinical experience with the minimally invasive resection of left-sided pancreatic cancer. Perhaps in the near future, more relevant clinical evidence with adequate long-term follow-up and qualified oncologic outcomes will become available, leading to the oncologic feasibility of minimally invasive left-sided pancreatectomy in pancreatic cancers. Generally, these conclusions will be influenced by selection bias from the retrospective nature of studies. However, these identified instances of selection bias, in turn, will become potential selection criteria for minimally invasive radical pancreatectomy in distal pancreatic cancers, especially given the difficulty of establishing an RCT in the present circumstances.

CONCLUSION

More than 20 years have passed since the first laparoscopic cholecystectomy was performed in the late 1980s. Tremendous improvements in the surgical techniques, experiences, and new effective instruments have successfully expanded the indications for laparoscopic surgery. Minimally invasive (laparoscopic and robotic) radical pancreatectomy in well-selected left-sided pancreatic cancers is feasible under general oncologic concepts; however, solid clinical evidence is still lacking. Further clinical experience with a minimally invasive approach to left-side pancreatic cancer must be carefully accumulated by experienced surgeons. The oncological feasibility should be addressed in greater detail based on long-term survival outcomes. However, we should not overlook that the currently available interim results demonstrating minimally invasive left-sided radical pancreatectomy are not inferior to those of conventional open radical pancreatectomy.

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2014 ADVANCES IN PANCREATIC CANCER

Optimum chemotherapy in the management of metastatic pancreatic cancer

Marwan Ghosn, Hampig Raphael Kourie, Fadi El Karak, Colette Hanna, Joelle Antoun, Dolly Nasr

Marwan Ghosn, Hampig Raphael Kourie, Fadi El Karak, Colette Hanna, Joelle Antoun, Dolly Nasr, Department of Oncology, Faculty of Medicine, Saint Joseph University, Beirut 166830, Lebanon
Author contributions: Ghosn M initiated the review; Ghosn M and Kourie HR performed the review and wrote and analyzed the data; Ghosn M, Kourie HR, El Karak F, Hanna C, Antoun J, and Nasr D reviewed and commented on the paper and provided final approval.
Correspondence to: Marwan Ghosn, MD, Department of Oncology, Faculty of Medicine, Saint Joseph University, Monot St, Beirut 166830, Lebanon. mghosn.hdf@usj.edu.lb
Telephone: +961-1-3226842　Fax: +961-1-1613397
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Core tip: This paper will be the newest study with the most recent updates in the treatment of metastatic pancreatic cancer. After a brief review of the different treatments for metastatic pancreatic cancer, the current treatment options are discussed, as well as novel therapies and approaches in the future.

Abstract

Pancreatic cancer is one of the most devastating solid tumors, and it remains one of the most difficult to treat. The treatment of metastatic pancreatic cancer (MPC) is systemic, based on chemotherapy or best supportive care, depending on the performance status of the patient. Two chemotherapeutical regimens have produced substantial benefits in the treatment of MPC: gemcitabine in 1997; and FOLFIRIONOX in 2011. FOLFIRINOX improved the natural history of MPC, with overall survival (OS) of 11.1 mo. *Nab*-paclitaxel associated with gemcitabine is a newly approved regimen for MPC, with a median OS of 8.6 mo. Despite multiple trials, this targeted therapy was not efficient in the treatment of MPC. Many new molecules targeting the proliferation and survival pathways, immune response, oncofetal signaling and the epigenetic changes are currently undergoing phase I and II trials for the treatment of MPC, with many promising results.

INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive and devastating solid tumors with the worst mortality. The median overall survival (OS) is less than 6 mo, and less than five percent of patients will survive more than 5 years (2% in cases of metastatic pancreatic cancer). The large majority of pancreatic cancers are locally advanced (50%) or metastatic (40%) because of their late diagnoses[1].

PC remains one of the most difficult cancers to treat due to its intrinsic resistance to conventional treatments. Many regimens have been implicated in the treatment of metastatic pancreatic cancers (MPC), but only two have had significant impact: GEMCITABINE, introduced in 1997[2]; and FOLFIRINOX, introduced in 2011[3]. In the

Table 1 Summary of the results of four trials associating Gemcitabine and targeted therapies

Ref.	Regimen	ORR	Median PFS (mo)	Median OS (mo)
Philip *et al*[10]	Gem/cetuximab	12.5%	3.4	6.3
	Gem	14.0%	3	5.9
Kindler *et al*[11]	Gem/bevacizumab	13.0%	3.8	5.8
	Gem	10.0%	2.9	5.9
Moore *et al*[12]	Gem/erlotinib	8.6%	3.75	6.24
	Gem	8.0%	3.55	5.91
Rougier *et al*[13]	Gem/aflibercept	ND	3.7	6.5
	Gem	ND	3.7	7.8

ORR: Objective response rate; PFS: Progression free survival; OS: Overall survival; ND: Not determined.

era of targeted therapy, the treatment of pancreatic cancer remains based mainly on chemotherapeutical regimens.

EVOLUTION OF TREATMENT MODALITIES

The primary goals of treatment in MPC are better quality of life, palliation and improved survival. The vast majority of chemotherapeutic drugs have been tried in the treatment of MPC, but few have been selected as standards of care.

Before the approval of GEMCITABINE, 5-FU was the most evaluated agent for MPC, without any survival amelioration. In 1997, Gemcitabine was approved by the FDA, based on the results of a randomized trial, in which Gemcitabine was compared to 5-FU in previously untreated patients. A total of 23.8% of Gemcitabine-treated patients experienced a clinical response, compared with 4.8% of 5-FU-treated patients ($P = 0.0022$), while the median survival was only extended by 1.24 mo (5.65 *vs* 4.41) in favor of patients receiving Gemcitabine ($P = 0.025$). The one-year survival rate was 18% for Gemcitabine patients and 2% for 5-FU patients[2].

Since the Gemcitabine era, many gemcitabine-based combination therapies have been widely evaluated over the past decade. Most trials have used a second cytotoxic agent, such as 5-FU[4], capecitabine[5], oxaliplatin[6], cisplatin[7], irinotecan[8] and pemetrexed[9], or a targeted therapy, such as cetuximab[10], bevacizumab[11], erlotinib[12] and aflibercept[13], administered in combination with gemcitabine (Table 1). However, despite a modest improvement in progression-free survival in some trials, a significant benefit in overall survival could not be demonstrated for the majority of these combination therapies.

Of all of these treatments, eroltinib, which positively impacted overall survival, was approved for the treatment of metastatic pancreatic cancer[10]; the addition of bevacizumab to gemcitabine-erlotinib did not lead to a statistically significant improvement in OS[14]. A trend toward better survival was also observed with a gemcitabine-capecitabine regimen. Finally, two meta-analyses, the first by Heinemann *et al*[15] and the second by Sultana *et al*[16], concluded that there was a significant survival benefit when gem-

citabine was associated with another agent (platinum and 5-FU derivatives) in patients with good performance status. A recent retrospective study by Khalil *et al*[17] in 2013 reported that adding erlotinib to gemcitabine-cisplatin did not appear to improve OS in MPC.

In 2007, we reported on a phase II clinical trial assessing a gemcitabine-free regimen based on FOLFOX 6, with promising results. A partial response was observed in 27.5% of the patients and stable disease in 34.5%[18]. Our study and the study by Louvet *et al*[6], which associated gemcitabine and oxaliplatine (RR of 26.8%, the highest with any gemcitabine-based regimen), highlighted the potential role of oxaliplatine in the treatment of MPC.

A second revolution marked the history of MPC in 2011, when Conroy *et al*[3] reported for the first time in NEJM a significant improvement in OS using a gemcitabine–free regimen-the FOLFIRIONOX regimen, based on three chemotherapeutic drugs: 5-FU, irinotecan and oxaliplatine. In this study, the median OS of the patients receiving FOLFIRINOX was 11.1 mo compared to 6.8 mo in the group of patients receiving gemcitabine alone, with an objective response rate of 31.6% compared to 9.4% in favor of the FOLFIRINOX arm. However, more adverse events, such as febrile neutropenia, thrombocytopenia, sensory neuropathy and diarrhea, were noted in the group of patients receiving FOLFIRINOX. This regimen was considered an option for the treatment of patients with MPC and good performance status[3]. A recent study demonstrated that FOLFIRINOX significantly reduced quality of life impairment compared with gemcitabine in patients with MPC[19].

Since the results with the FOLFIRINOX gemcitabine-free regimen, a new attempt with gemcitabine-based combination therapy revealed promising results. Another agent added to gemcitabine was the *nab*-paclitaxel, an albumin-bound nanoparticle form of paclitaxel that increases the tumor accumulation of paclitaxel through binding of albumin to SPARC. A randomized phase III study that compared a combination of *nab*-paclitaxel and Gemcitabine weekly to gemcitabine alone showed a significant improvement in overall survival of 8.5 mo *vs* 6.7 mo ($P < 0.05$) and a response rate of 23% *vs* 7%[20]. An important prognostic biomarker in patients with MPC receiving *nab*-paclitaxel is SPARC; a positive SPARC status in these patients was associated with a significant increase in OS[21].

CURRENT TREATMENT OPTIONS

Treatment is systemic, based on chemotherapy or best supportive care, depending on the performance status of the patient.

In patients with limited performance status, Gemcitabine as monotherapy is the uniquely approved treatment; another alternative is best supportive care. In patients with good performance status, many chemotherapeutical regimens are available (Table 2). Gemcitabine is still considered a possible option[1]. FOLFIRINOX offers the best overall survival and response rate in MPC, but it causes many side effects. Gemcitabine associated

Table 2 The approved chemotherapeutical regimens for metastatic pancreatic cancer in patients with good performance status

Ref.	Regimen	ORR	Median OS (mo)	Median PFS (mo)
Burris *et al*[2]	Gemcitabine	ND	5.65	2.33
	5-FU	ND	4.41	0.92
Conroy *et al*[3]	FOLFIRINOX	31.6%	11.1	6.4
	Gemcitabine	9.4%	6.8	3.3
Moore *et al*[12]	Gemcitabine/ erlotinib	8.6%	6.24	3.75
	Gemcitabine	8.0%	5.91	3.55
Daniel *et al*[20]	Gemcitabine/ *nab*-paclitaxel	23%	8.5	5.5
	Gemcitabine	7%	6.7	3.7

ORR: Objective response rate; OS: Overall survival; PFS: Progression free survival; ND: Not determined.

Table 3 The adverse events of three approved regimen for metastatic pancreatic cancer reported in NEJM 2011 and ASCO 2013

Adverse events	Gemcitabine	FOLFIRINOX	Gemcitabine/ *nab*-paclitaxel
Neutropenia	21%	45.7%	38%
Febrile neutropenia	1.2%	5.4%	3%
Thrombocytopenia	3.6%	9.1%	13%
Fatigue	17.8%	23.6%	17%
Diarrhea	1.8%	12.7%	6%
Peripheral neuropathy	0%	9.0%	17%

with *nab*-paclitaxel offers the second best overall survival, with fewer side effects compared to FOLFIRINOX[16,18]. A comparison between the side effects of these three regimens is resumed in the Table 3. Erlotinib remains the unique targeted therapy approved for the treatment of MPC in combination with gemcitabine. Gemcitabine combined with cisplatin or capecitabine can be a reasonable choice in some cases. Patients with MPC and good performance status can also be included in different phase I or II clinical trials. All of the approved treatments for MPC in patients having poor and good performance status are reviewed in Figure 1.

The second-line treatment for MPC has been evaluated in only a few trials. The general guidelines for treatment are to use fluoropyrimidine-based chemotherapy if the patient was previously treated with gemcitabine-based chemotherapy and gemcitabine-based chemotherapy if previously treated with fluoropyrimidine-based therapy[22]. A phase II trial investigated whether the association of capecitabine with oxaliplatin was active in gemcitabine-pretreated patients with MPC, especially patients with a good performance status and those who responded to first-line chemotherapy[23]. A phase III trial comparing the OFF regimen (oxaliplatin; 5-FU; folinic acid) to best supportive care provided first-time evidence for the benefit of second-line chemotherapy in MPC, manifested by prolonged survival time[24]. Palliative radiotherapy has been proposed as salvage therapy for patients with severe pain refractory to narcotics[22].

Novel therapies and approaches

Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have been considered, for the last decade, the two main targets that should be studied in MPC. Many trials have combined gemcitabine with an anti-angiogenic drug or a tyrosine-kinase inhibitor (Table 1); all of these trials have had negative results, except for the combination of gemcitabine and erlotinib, as mentioned above.

After multiple failures with targeted therapy for MPC based on anti-EGFR and anti-VEGF, many new con-

cepts for treating MPC are being elaborated, including the targeting of tyrosine kinase signaling, cascade elements, the stromal reaction, the immune response, oncofetal signaling and epigenetic changes[25].

IFG1R, MEK, PI3K, AKT, and mTOR are actually the most frequent signaling pathway targets evaluated in the treatment of MPC. A phase II trial reported that ganitumab (AMG 479), an mAb antagonist of insulin-like growth factor 1 receptor, combined with gemcitabine showed a trend toward improved 6-mo survival and overall survival rates[26]. Many other trials had negative results: selumetinib (AZD6244), a selective MEK inhibitor compared to capecitabine as a second-line treatment after gemcitabine, did not demonstrate any statistically significant difference in overall survival[27]; an oral m-TOR inhibitor (RAD001) had minimal clinical activity in gemcitabine-resistant MPC[28].

Immunotherapy is one of the promising new concepts introduced in the treatment of MPC. A phase I study of an agonist of CD40 monoclonal antibody (CP-870, 893), in combination with gemcitabine, was well tolerated in patients with MPC and was associated with anti-tumor activity[29]. Ipilimumab (anti-CTLA-4), another immunotherapeutic option approved for metastatic melanoma[30], was considered ineffective in the treatment of MPC after the results of a phase II trial; association of these agents with other agents could probably have more promising results[31].

Another approach in the treatment of MPC is the targeting of oncofetal signaling, which is responsible for tumor progression and resistance to chemotherapy in PC. One of the most altered pathways incriminated in the development of PC is the Notch pathway[32]; the activation of γ-secretase is the primum movens of activation of Notch signaling. Preclinical data suggested that a selective γ-secretase inhibitor (PF-03084014) had greater anti-tumor activity in combination with gemcitabine in PC, providing a rationale for further investigation of this combination in PC[33]. Many other trials are evaluating agents targeting the stromal reaction and epigenetic changes[34,35].

Another targeted therapy, AGS-1C4D4, a fully human monoclonal antibody against prostate stem cell antigen, was evaluated with gemcitabine in a randomized phase II study of untreated MPC, with achievement of its primary end point in demonstrating improved 6-mo SR[36]. All of the recent phase II trials studying the new agents in the
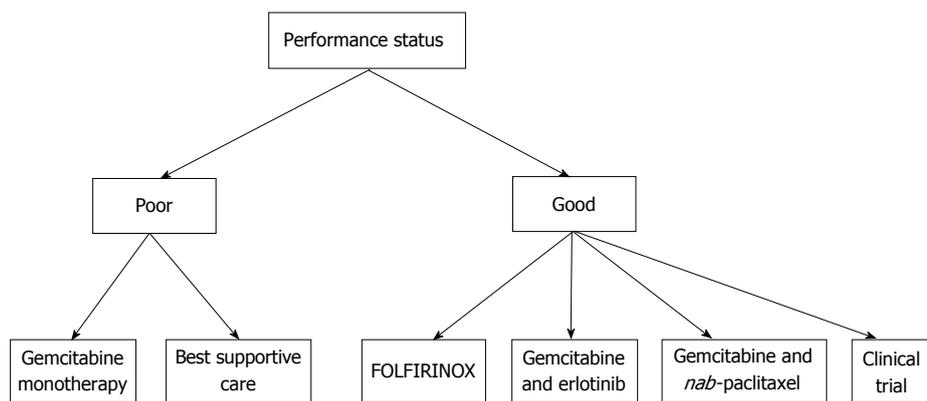
Figure 1 A schema representing the approved treatment for metastatic pancreatic cancer in patients having poor or good performance status.

Table 4 Recent phase Ⅱ trials studying new agents in the metastatic pancreatic cancer					
Reference	New agents	Agents target	Phase of the study and targeted population	Arms of the study	Conclusion of the study
Kindler *et al*[26]	Ganitumab (AMG479)	mAb antagonist of insulin-like growth factor 1 receptor	Phase Ⅱ; untreated MPC patients	Gem/ganitumab *vs* gem	Improved 6-mo survival rate and OS
Bodoky *et al*[27]	Selumetinib (AZD6244)	Selective MEK inhibitor	Phase Ⅱ; second line treatment after gemcitabine	Selutimumab *vs* capecitabine	No significant difference in OS
Wolpin *et al*[28]	Everolimus (RAD001)	m-TOR inhibitor	Phase Ⅱ; second line treatment after gemcitabine	Everolimus (single arm study)	Minimal clinical activity
Royal *et al*[31]	Ipilimumab (MDX010)	Anti-CTLA4	Phase Ⅱ; untreated MPC patients	Ipilimumab (single arm study)	Ineffective in the treatment of MPC
Wolpin *et al*[36]	AGS-1C4D4	mAb to prostate stem cell Antigen	Phase Ⅱ; untreated MPC patients	Gemcitabine/AGS-1C4D4 *vs* gemcitabine	Improved 6-mo survival rate

MPC: Metastatic pancreatic cancer; OS: Overall survival.

treatment of MPC are summarized in Table 4.

Many new targets and genes that play roles in the pathogenesis and progression of PC are being evaluated in animals or in cancer cells for their potential diagnostic and therapeutic implications: mucin (myc) was studied by Rachagani *et al*[37], transketolase by Wang *et al*[38] and aberrant CD20 expression by Chang *et al*[39].

The combination of these novel therapies and approaches could positively affect the history of MPC.

CONCLUSION

Despite multiple trials and their major efforts, PC remains resistant to chemotherapy and targeted therapy. It seems that the results obtained with chemotherapy, targeted therapy and their combination in MPC have reached a plateau, with significant, but modest, amelioration of OS of less than one year. Stratified or personalized therapy is totally absent in the treatment of MPC, due to the absence of prognostic or therapeutic markers and the lack of molecular profiling modalities. Many trials are currently being conducted to explore new targets in the tumorigenesis and proliferation of PC. Finally, the combination of these novel therapies with personalized medicine might offer promising results in patients with MPC.

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World Clinical Pancreatology

2014 ADVANCES IN PANCREATIC CANCER

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Screening and early detection of pancreatic cancer in high risk population

Ming-Chu Chang, Jau-Min Wong, Yu-Ting Chang

Ming-Chu Chang, Jau-Min Wong, Yu-Ting Chang, Department of Internal Medicine, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei 101, Taiwan

Author contributions: Chang MC, Wong JM and Chang YT designed the study; Chang MC and Chang YT wrote the manuscript.

Correspondence to: Yu-Ting Chang, MD, MS, PhD, Department of Internal Medicine, National Taiwan University Hospital, No.7, Chung Shan South Road, Taipei 101, Taiwan. yutingchang@ntu.edu.tw

Telephone: +886-2-23123456 Fax: +886-2-23633658

Abstract

Pancreatic cancer is a serious growing health issue in developed countries. For patients diagnosed with pancreatic cancer, the five year survival rate is below 5%. One major important reason leads to the poor survival rate is lack of early detection of pancreatic cancer. Over 80% of the patients are diagnosed in advanced disease stages. Screening for pancreatic cancer is a desirable option for high risk individuals to allow early detection and treatment of curable pancreatic neoplasms at a pre-invasive stage. This article highlights the need, endpoint, population, method, diagnostic yield, and the problems of current screening programs.

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tion and treatment of curable pancreatic neoplasms at a pre-invasive stage. This article highlights the need, endpoint, population, method, diagnostic yield, and the problems of current screening programs.

INTRODUCTION

Pancreatic cancer is one of the most lethal diseases despite marked improvement in medical and cancer care over the past years. The number of newly diagnosed pancreatic cancer patients has increased significantly in recent years[1]. The most common histological subtype of pancreatic cancer is adenocarcinoma, which comprises 87% of the pancreatic malignancies. Among the pancreatic cancer patients, there were only 15%-20% diagnosed as "resectable" and surgery was the only way to treat the disease. The majority of the pancreatic cancer patients were diagnosed as unresectable and chemotherapy was the standard treatment to control the incurable disease. The prognosis for patients with pancreatic cancer remains poor. The overall survival rate was 5% combining all stages, 20% for patient with localized disease and 1%-2% for those with distant metastasis. In most cases, pancreatic cancer has progressed before clinical manifestation. Many patients initially thought to have localized and resectable cancer succumb to recurrent or metastatic disease. Hence, there is an urgent need to detect small asymptomatic cancers or precursor lesions, which are potentially curable for the most devastating disease.

OPPORTUNITY AND POTENTIAL WINDOW OF SCREENING

A recent study suggested that there may be a large window and good opportunity for detecting pancreatic cancer when the disease is in earliest and most treatable stages[2]. Quantitative analysis of the timing of the genetic evolution of sporadic pancreatic cancer indicated a time span of at least 10 years between the occurrence of cancer-initiating mutations and the formation of parental nonmetastatic founder cell[2]. Indeed, patients with pancreatic tumors diagnosed incidentally had longer median survival[3] than those with tumors discovered after symptoms appeared, suggesting that early detection of small asymptomatic cancers or precursors lesions may improve the outcome. Identification of high risk populations of pancreatic cancer for screening becomes essential. Distinct clinical and genetic features are thought to increase the risk of pancreatic cancers. It has been estimated about 10% of pancreatic cancer has a familial basis. Hereditary pancreatic cancer includes inherited cancer syndromes with a recognized known germline mutation associated with an increased risk of pancreatic cancer and familial pancreatic cancer with two or more cases of pancreatic cancer in their families. Screening pancreatic cancer in these high risk individuals might be recommended for early detection to improve the prognosis of pancreatic cancer. A multidisciplinary international consortium met to discuss pancreatic screening was held recently[4] and some statements regarding to pancreatic cancer screening were made and voted to guide the pancreatic cancer screening.

DEFINITION OF "SUCCESS" OF SCREENING

A very recent effort made by international cancer of the pancreas screening (CAPS) has proposed to define "successful screening" by detection and treatment of T1N0M0 margin negative pancreatic cancer and high grade dysplastic precursor lesions, including pancreatic intraepithelial neoplasia-3 (PanIN-3), intraductal papillary mucinous neoplasm (IPMN) with high grade dysplasia, and mucinous cystic neoplasm (MCN) with high grade dysplasia[4].

WHO TO SCREEN?

Not all populations with risk of pancreatic cancer need to be screened because there is no evidence that screening for pancreatic cancer is effective in reducing mortality and the harms of screening for pancreatic cancer exceed any potential benefits[5]. Clinical risk factors include age, obesity, smoking, diabetes, and non-genetic chronic pancreatitis are associated with pancreatic cancer. However, the specificity of these factors to pancreatic cancer is low. For example, the risk for developing pancreatic cancer increases with age, mostly in individuals at age over 45.

Overweight and obese individuals have an increased risk (odds ratio: 1.8 and 1.22 in males and females, respectively) and earlier disease onset[6]. Current cigarette smokers and former smokers who had quit for less than 5 years also have a higher risk of pancreatic cancer than non-smokers (odds ratio: 1.71 and 1.78 for current smokers and recent past smokers, respectively)[7]. Patients with diabetes are at higher risk for pancreatic cancer (odds ratio: 1.76)[8], and new onset of diabetes may be an early indicator of pancreatic cancer[9]. Several studies have indicated that patients with (non-genetic)chronic pancreatitis had a higher incidence of pancreatic cancer over the general population (odds ratio: 2.23)[10-12].

HIGH RISK POPULATIONS

Screening is suggested in high risk populations, including individuals with lifetime risk of pancreatic cancer over 5% or/and increased relative risk over 5 times proposed by CAPS[4]. Table 1 listed the proposed high risk population to screen and their relatively risk and/or lifetime risk of pancreatic cancer.

PEUTZ-JEGHERS SYNDROME

Peutz-Jeghers (PJ) syndrome is an autosomal dominantly inherited syndrome caused germline *STK11* gene mutations with high penetrance[13]. It is characterized by mucocutaneous pigmentation and hamartomatous polyps of the gastrointestinal (GI) tract. Patient with PJ syndrome have a risk of multiple GI and non-GI cancers. The cumulatively lifetime risk of pancreatic cancer is 36%, with a relatively risk (RR) of 132[14,15]. Patients with PJ syndrome whatever with family history of pancreatic cancer are suggested to be candidates for pancreatic cancer screening[4].

FAMILIAL ATYPICAL MULTIPLE MOLE MELANOMA SYNDROME

Familial atypical multiple mole melanoma syndrome is an autosomally dominant disease with variable penetrance caused by *p16/CDKN2A* gene mutation[16]. It is characterized by familial occurrence of multiple benign melanocytic nevi, dysplastic nevi, and melanoma[17]. Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome is associated with extrapancreatic (sarcomas, endometrial, breast and lung cancers) and pancreatic cancers. The risk of developing pancreatic cancer risk is about 13-22 folds[18-20]. *p16* mutation carriers with one or more affected first degree relative (FDR) with pancreatic cancer should be considered for screening[4].

FAMILIAL BREAST AND OVARIAN CANCER

Familial breast and ovarian cancer syndrome is an autosomal dominantly inherited syndrome associated

Table 1 High risk population and the estimated risk for pancreatic cancer

Syndrome	Gene	RR	Lifetime risk
Peutz-Jeghers syndrome	STK11/LKB1	132	36% by age 65 yr
Hereditary pancreatitis	PRSS1	53	Male: 11% and 49% by age 50 and 75 yr Female: 8% and 55% by age 50 and 75 yr
FAMMM	p16	13-22	16% lifetime risk
Familial breast and ovarian	BRCA1/2	3-10	5% lifetime risk
HNPCC	MLH1, MSH6, MSH2, PMS2	1.5-9	8.6% lifetime risk
Familial pancreatic cancer			
2 FDR	Unknown	6.4	8%-12% lifetime risk
3 FDR	Unknown	32	40% lifetime risk

FDR: First degree relative; HNPCC: Hereditary non-polyposis colorectal cancer; PRSS1: Cationic trypsinogen gene; FAMMM: Familial atypical multiple mole melanoma syndrome.

with germline mutations of *BRCA1* and *BRCA2* genes. Mutation carriers are at high risk for breast, ovarian, GI cancers (bile duct, gallbladder, stomach, pancreas) and prostate cancers[21-24]. BRCA2 carriers are associated with higher risk of pancreatic cancer (3-10 folds) than BRCA1 carriers (2.3-3.6 folds)[24,25]. *BRCA2* mutation carriers with one or more affected FDR with pancreatic cancer and those with two or more affected family members (even without a FDR) should be considered for screening[4].

HEREDITARY NON-POLYPOSIS COLORECTAL CANCER, LYNCH SYNDROME

The lynch syndrome is associated with mismatch repair genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*). Hereditary non-polyposis colorectal cancer (HNPCC) is characterized by early-onset colorectal cancers and extra-colonic cancers including pancreas[26]. The lifetime risk of pancreatic cancer is 3.7% by age of 70, an 8.6-fold increased risk compared to general population[27]. Patients with Lynch syndrome and one affected FDR with PC should be considered for screening[4].

HEREDITARY PANCREATITIS

Hereditary pancreatitis is a rare inherited disorder. It is transmitted as an autosomal dominant disorder with incomplete penetrance[28,29]. Hereditary pancreatitis is associated with a high risk of pancreatic cancer with a lifetime risk about 40%[30]. In those individuals with a paternal inheritance pattern, the cumulative risk is even approaching 75%[30]. This risk of pancreatic cancer is related to the duration of inflammation[31]. Screening of *PRSS1* (Cationic trypsinogen) mutation carriers with longstanding

chronic pancreatitis is being performed within established programs[32].

FAMILIAL PANCREATIC CANCER

Familial pancreatic cancer (FPC) describes families with at least two first-degree relatives with confirmed exocrine pancreatic cancer that do not fulfill the criteria of other inherited tumor syndromes. FPC is also used to describe families with exocrine pancreatic cancer in two or three or more relatives of any degree[33,34]. An indicative pattern of an autosomal dominant trait of inheritance has been identified in 58%-80% of FPC families[35-37]. Previous studies have described an increased risk of developing pancreatic cancer in unaffected FDRs that depends on the number of relatives with pancreatic cancer[38]. Studies of the European Registry of Hereditary Pancreatitis and FPC (EUROPAC) and German national case collection for FPC (FaPaCa) described the phenomenon that patients in younger generations develop the disease about 10 years earlier than their affected parents[36,37]. The proportion of patients younger than 50-year-old appeared to be higher (16%) in FPC families compared to the general population[34]. For individuals with two affected first-degree relatives and individuals with three affected first-degree relatives, the relative risk are 32[39]. The risk of pancreatic cancer seemed to be higher among members of FPC kindred with a young age of onset (younger than 50 years of age) compared with kindred with an age of onset older than 50 years of age. The life-time risk rose to 38% for individuals with three affected first-degree relatives, if one of the affected was diagnosed under the age of 50 years[40].

PALB2 gene was identified as a PC susceptibility gene recently[41]. It is a partner and localizer of *BRCA2*. *PALB2* germline mutations have been detected in up to 3% of patients with familial PC[41-43]. The risk of PC among *PALB2* gene mutation carriers is estimated to be similar to that found for *BRCA2* gene mutation carriers. *PALB2* mutation carriers with one or more affected FDR with PC should be screened[4].

WHEN TO SCREEN?

Patients with hereditary pancreatitis has an higher risk of early onset pancreatic cancer. Screening typically begins at age 40 in *PRSS1* mutation carriers[44]. In other high risk populations, there is no consensus as to whether to recommend initiating screening and the end of screening[4].

SCREEN TECHNIQUES

Up to now, there is no ideal single screening method or screening program for detection of early pancreatic cancer. Serum CA19-9 levels is the most commonly used serum marker in pancreatic cancer. However, the sensitivity and specificity of serum CA19-9 as a diagnostic marker are not good for screening pancreatic

Table 2 Reported pancreatic cancer screening programs and diagnostic yield

Study	Screening modalities	Case (*n*)	Study population	Diagnostic yield upon imaging
Rulyak *et al*[46], 2001	EUS	35	FPC	34.3%
Canto *et al*[56], 2004	EUS	38	FPC, PJS	76%
Canto *et al*[55], 2006	EUS	78	FPC, PJS	22%
Poley *et al*[47], 2009	EUS	44	FPC, FAMMM, PJS	23%
Langer *et al*[48], 2009	EUS + MRCP	76	FPC, PCMS	36%
Verna *et al*[49], 2010	EUS and/or MRCP	51	FPC, FAMMM, HNPCC	EUS: 65% MRI: 33%
Ludwig *et al*[50], 2011	MRCP	109	FPC	8.3%
Vasen *et al*[51], 2011	MRCP	79	FAMMM	20%
Canto *et al*[52], 2012	MRCP, EUS, CT	216	FPC, HBOC, PJS	42.6%
Al-Sukhni *et al*[53], 2012	MRCP	262	FPC, FAM-MM, PJS, hereditary pancreatitis	32%

EUS: Endoscopic ultrasonography; MRI: Magnetic resonance imaging; CT: Computed tomography; FAMMM: Familial atypical multiple mole melanoma syndrome; FPC: Familial pancreatic cancer; HBOC: Hereditary breast-ovarian cancer; HNPCC: Hereditary nonpolyposis associated colorectal cancer; IPMN: Intraductal papillary mucinous neoplasia; MRCP: Magnetic resonance cholangiopancreatography; PCMS: Pancreatic carcinoma-melanoma syndrome; PJS: Peutz-Jeghers syndrome.

cancer[45]. The most common screening imaging used for the detection of pancreatic cancer are endoscopic ultrasonography (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP). EUS is an invasive procedure which might detect lesions smaller than 1 cm. However, the major problem of EUS is its operator dependent. CT scanning demonstrated a low sensitivity to detection pancreatic dysplasia. MRI with MRCP is a non-invasive procedure which could detect earlier ad minor changes in pancreatic parenchymal and (main) pancreatic duct compared to CT scan. Table 2 summarized the reported pancreatic cancer screening programs with the reported diagnostic yield. MRI with MRCP and EUS are considered the most accurate tools for pancreatic imaging as promising recommended tool for screening[46-56]. The major weakness of CT is its radiation exposure and the suboptimal detection rate as a routine screening tool for asymptomatic high risk individuals[4]. MRI with MRCP is less invasive and more objective compared to EUS. It still lacks randomized controlled studies to compare EUS and MRI with MRCP in pancreatic cancer screening in high risk individuals. Regarding to the imaging study as a screening tool, over diagnosis is a major problem which might cause over treatment of a benign lesion. The risk of incorrect diagnosis is particularly high for EUS because of it is an operator-dependent examination with only modest interobserver agreement[57]. Abdominal ultrasound and endoscopic retrograde cholangiopancreatography are not recommended for screening, owing to their low diagnostic sensitivity and the risk of pancreatitis, respectively[4].

EMERGING PROBLEMS AND FUTURE PROSPECTIVE

There are still some unresolved problems in pancreatic cancer screening. First of all, the aim of screening is to find the earliest pancreatic cancer (T1N0M0) or high grade precursor lesions in PanIN, IPMN and MCN. In fact, the high grade PanINs are actually microscopic lesions which might cause some tiny or abnormal findings in imaging. Even with fine needle aspiration, the aspirated substance could not represent the worst condition or whole picture what it is. Secondly, we still have no imaging modality or accurate criteria to differentiate benign pancreatic cystic lesions from malignant cystic tumors with dysplasia or malignancy. There are some proposed "high risk stigmata and worrisome features"[58] to help us for picking up true meaningful or suspected malignant pancreatic cystic lesions or IPMN to avoid unnecessary operations or overtreatment. However, there is still no reliable or good method to different the nature of pancreatic cystic lesions. With the advancement and frequent use of abdominal imaging, more and more incidentally found pancreatic lesions and/or IPMNs are disclosed. How to follow up the increasing numbers of patient with optimal programs to avoid under detection of pancreatic cancer and also to avoid overtreatment will be a great challenge for clinician.

CONCLUSION

Screening pancreatic cancer in high risk populations is suggested to enhance the potential early detection of curable early pancreatic cancer. It is a potential way to improve the outcome of pancreatic cancer. Although some consensus are proposed to be followed, there is still lack of ideal screening method and program at the present time. Further study and advancement for improving the sensitivity and specificity of screen methods to achieve the goal of early detection of pancreatic cancer is warranted in the near future.

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2014 ADVANCES IN PANCREATIC CANCER

Laparoscopic resection of pancreatic neuroendocrine tumors

Abbas Al-Kurd, Katya Chapchay, Simona Grozinsky-Glasberg, Haggi Mazeh

Abbas Al-Kurd, Katya Chapchay, Haggi Mazeh, Department of Surgery, Hadassah-Hebrew University Medical Center, Mount Scopus, Jerusalem 91240, Israel

Simona Grozinsky-Glasberg, Neuroendocrine Tumor Unit, Hadassah-Hebrew University Medical Center, Mount Scopus, Jerusalem 91240, Israel

Author contributions: Grozinsky-Glasberg S, Mazeh H contributed the conception of the study; Al-Kurd A, Chapchay K, Mazeh H contributed to the literature review and data acquisition; Al-Kurd A, Chapchay K drafted the manuscript; Chapchay K contributed illustrations; Al-Kurd A, Chapchay K, Grozinsky-Glasberg S, Mazeh H approved the final version.

Correspondence to: Haggi Mazeh, MD, Department of Surgery, Hadassah-Hebrew University Medical Center, Mount Scopus, PO Box 24035, Jerusalem 91240, Israel. hmazeh@hadassah.org.il

Telephone: +11-972-25844550 Fax: +11-972-25844584

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Abstract

Pancreatic neuroendocrine tumors (PNETs) are a rare heterogeneous group of endocrine neoplasms. Surgery remains the best curative option for this type of tumor. Over the past two decades, with the development of laparoscopic pancreatic surgery, an increasingly larger number of PNET resections are being performed by these minimally-invasive techniques. In this review article, the various laparoscopic surgical options for the excision of PNETs are discussed. In addition, a summary of the literature describing the outcome of these treatment modalities is presented.

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Key words: Pancreatic neuroendocrine tumor; Laparoscopy; Surgery; Laparoscopic resection of gastrointestinal

Core tip: Pancreatic neuroendocrine tumors (PNETs) are a rare clinical entity, with surgery being the treatment modality of choice. Over the past several years, laparoscopic techniques have gained popularity in the surgical management of these tumors. This article reviews the available literature on laparoscopic resection of PNETs, with an overview of the commonly-practiced surgical procedures.

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs), also known as islet cell tumors, are a rare form of endocrine neoplasms, accounting for a reported 1%-4% of all pancreatic tumors[1-3]. These tumors are associated with an annual incidence of one per 100000 population, and their diagnosis has increased over the past 40 years, most likely due to advances in imaging and histopathological methods[4-6]. PNETs can manifest at any age, however, most present during the 4th to 6th decades of life. When considered as a general entity, no gender predilection is demonstrated, but the various subtypes when observed separately do show slight gender predilection[7]. Although the majority of cases are sporadic, 10%-30% have been shown to be associated with multiple endocrine neoplasia (MEN) 1 syndrome, and < 1% with (Von Hippel-Lindau) VHL disease[8,9]. Other genetic syndromes in which PNETs may present include neurofibromatosis type 1 and tuberous sclerosis[4]. PNETs can be classified as functional and

nonfunctional, the latter being far more common and typically presenting late during disease evolution, with symptoms related to mass effect, invasion into surrounding structures, or metastasis[3].

The most common form of functional PNET is insulinoma, accounting for 70%-80% of cases. Ninety percent are benign and solitary, and they are predominantly located in the body and the tail of the pancreas (65%-80%). Due to the fact that symptoms of hypoglycemia dominate the clinical picture early in the course of the disease, the majority are small in size (< 2 cm) at the time of presentation, and compatible with surgical resection[10-12]. This contrasts with gastrinomas, another type of functional PNET, which in more than 50% are extrapancreatic, tend to be larger in size, and present with metastasis in 60%-70% of cases. Other rare types include glucagonomas and vasoactive intestinal peptide-producing tumors (VIPomas), the majority of which are also malignant (80% and 60%, respectively). Somatostatinomas are typically large neoplasms, causing mass effect around the pancreatic head or periampullary region. The majority of these tumors are malignant (70%)[13].

It is due to the above-mentioned characteristics that the "non-insulinoma PNETs" are less suitable for surgical resection. That said, surgery remains the only curative modality for neuroendocrine neoplasms of the pancreas, and is the treatment of choice when technically feasible, even in the presence of malignancy and occasionally locally advanced or metastatic disease[14-19].

With advances in minimally invasive surgery, laparoscopic resection has become a well-accepted modality in the management of pancreatic tumors, with an increasing number of surgeons utilizing these techniques[18]. The use of laparoscopy in pancreatic surgery was initially introduced in 1994 by Gagner *et al*[20] and Cuschieri[21], and two years later, Gagner *et al*[22] reported on their early experience with laparoscopic resection of islet cell tumors. Since then, several publications have described laparoscopic pancreatic surgery, however, only a small number of large series have described laparoscopic surgery in the setting of PNETs[13,18,23,24]. The purpose of this article is to review the available literature on laparoscopic resection of PNETs, with a focus on the various surgical techniques, and compare laparoscopic surgery to open pancreatic surgery in terms of results and complications.

PREOPERATIVE LOCALIZATION

Preoperative localization of the neuroendocrine tumor is of utmost importance in the management of these neoplasms. Prior to considering laparoscopy, an expected surgical strategy must be contemplated in accordance with the findings on imaging studies.

Imaging studies provide information regarding the location of the tumor, the extent of local invasion, and the presence of metastatic lesions[7]. Localization studies commonly used include ultrasonography, computed tomography (CT) scanning, and magnetic resonance imaging (MRI). CT is generally the initial test used by clini-

cians to localize PNETs. On CT scans, these tumors typically appear hyperdense on arterial phase. Although there is great variation in the literature regarding the reported usefulness of this modality for the detection of PNETs, it is generally accepted that it has a sensitivity of less than 50%-60%[25-27]. Nevertheless, one study reported a sensitivity of 94% for CT in the detection of PNETs[28]. MRI has the advantage of decreased radiation when compared with CT, and is commonly used to detect small PNETs and to assess local invasion[29]. A sensitivity ranging from 30% up to 95% has been reported in the literature for the detection of PNETs[30,31].

A study that has gained popularity due to increased accuracy is endoscopic ultrasound (EUS), however, the disadvantage of this technique is operator dependence[32-35]. It has a higher success rate in localizing tumors of the head and body than those of the tail. This modality has been associated with a sensitivity of 80% to 88% and a specificity of 95%[13]. One study reported a sensitivity of 82% and a specificity of 95% for EUS in the localization of PNETs not identified by CT or angiography[36]. It is also worth noting that the combination of EUS with biphasic helical CT scanning has been demonstrated to increase the diagnostic accuracy to 97%[13]. Although EUS has been shown to be effective in the detection of regional lymph node involvement, its usefulness in the diagnosis of liver metastasis is largely limited[37].

Angiographic techniques with portal vein sampling are invasive methods, and are typically reserved for patients in whom other less invasive diagnostic tests have failed to localize the pathology. These methods have been shown to provide accurate regionalization (but not exact localization) in up to 90% of cases[38].

A functional study commonly utilized is somatostatin receptor scintigraphy (octreotide scan)[39]. A relatively new modality shown to be superior to the octreotide scan in the diagnosis of neuroendocrine tumors is gallium-68 somatostatin receptor PET scan, which utilizes radio-labeled tracers with affinity to somatostatic receptors to localize these tumors[40]. A recently published meta-analysis demonstrated that this imaging modality has a sensitivity of 93% (when ^{68}Ga-DOTATOC is utilized) and 96% (when ^{68}Ga-DOTATATE is utilized). The specificity was shown to be 85% and 100%, respectively[41]. It should be noted that the diagnostic yield of these tests is reduced in insulinomas, which may not express somatostatin receptors. The use of FDG-PET CT for the diagnosis of PNETs is limited, mainly due to the slow-growing nature of these tumors[42]. However, the ability of this test to detect more aggressive tumors (due to the fact that less differentiated tumors consume more glucose) has been proposed[43].

It appears that after establishing the localization of a lesion by more than one noninvasive study (for example, CT scan and MRI), it is reasonable to explore the patient laparoscopically, and to perform an intraoperative ultrasound (discussed below)[44]. Due to the relative safety and diagnostic accuracy of modern laparoscopic techniques along with the use of intraoperative ultrasound, many

recommend that the utilization of more invasive pre-operative diagnostic methods, such as angiography, be reserved for equivocal cases only[13].

DETERMINATION OF SURGICAL TECHNIQUE

Surgery remains the cornerstone of management of PNETs, with increased utilization of the laparoscopic approach demonstrated over the past two decades[45]. The planned surgical approach is governed largely by the findings in preoperative localization studies, but may commonly change in accordance with intraoperative findings.

There is no general consensus regarding the indications for and limitations of laparoscopic surgery for PNETs. Although some have claimed that the presence of a malignant PNET is a contraindication for laparoscopic resection[46], others have shown the feasibility and safety of laparoscopic surgery in these malignant tumors[24].

Laparoscopic enucleation is utilized in lesions less than 3 cm in size which are noninvasive and located peripherally and thereby do not involve the main pancreatic duct. When the above criteria are fulfilled, this procedure may be applicable for tumors located in the pancreatic head, body, or tail[47]. When the tumor is in proximity to the Wirsung duct, enucleation is not suitable due to the elevated risk of pancreatic fistula development[48]. Due to the fact that insulinomas are typically small, single and benign lesions, the use of laparoscopic enucleation for surgical management of these tumors has been widely described in the literature. The use of intraoperative ultrasound, however, is essential in order to rule out the presence of other lesions before the decision to perform enucleation is made, and to assess the proximity of these tumors to the pancreatic duct and vascular structures[48].

When the PNET is not compatible with enucleation, pancreatic resection is necessary[47]. In tumors involving the head of the pancreas, pancreaticoduodenectomy (Whipple Operation) is indicated. This procedure is not widely performed laparoscopically worldwide, due to the associated technical difficulties. However, many studies have shown the effectiveness and safety of this procedure, when performed by sufficiently-trained hepatobiliary or laparoscopic surgeons[49-52].

In tumors that are located in the body or the tail and are not suitable for enucleation, laparoscopic distal pancreatectomy is the treatment of choice. This surgery can be further divided into three different entities: spleen-preserving distal pancreatectomy with splenic vessel preservation, spleen-preserving distal pancreatectomy without splenic vessel preservation, and distal pancreatectomy with splenectomy[53]. The main factors which dictate the procedure chosen are the location of the tumor within the pancreatic body or tail, and its relation to the splenic vessels and splenic hilum. In addition, the presence or suspicion of malignant neuroendocrine tumors generally favors more radical approaches, with the resection of splenic vessels in order to enable adequate lymph node sampling. Ligation of splenic vessels is also advocated when uncontrolled bleeding from the vessels at the upper border of the pancreas is demonstrated intraoperatively[13]. This procedure is less technically demanding and is associated with shorter operation time. Ligation and transection of the splenic vessels is performed at the level of the pancreatic resection and at the splenic hilum. Postoperatively, the spleen receives its vascular supply from the short gastric vessels and left gastroepiploic vessels[13].

When possible, spleen-preserving distal pancreatectomy with splenic vessel preservation is performed; however, this procedure requires higher technical expertise, with separation of the splenic vessels from the pancreatic parenchyma, and the dissection and ligation of the branching vessels supplying the pancreas. As a result, this procedure is associated with a longer operating time[54-57]. Splenic preservation in these PNETs is generally encouraged when it is technically feasible; however, the occasional presence of hilar fibrosis due to previous inflammation can make splenic preservation difficult, and in these cases, *en bloc* pancreaticosplenectomy appears to be the safest option[58]. This is also true in malignant PNETs that involve or are adjacent to the hilum of the spleen and in these cases, the need for a complete oncologic resection supersedes the benefits of splenic preservation. That said, the avoidance of splenectomy can be achieved in the majority of cases[54-56]. In Assalia and Gagner's publication, the rate of successful splenic salvage in laparoscopic distal pancreatectomy for islet cell tumors approached 85%[13].

It is worth mentioning that in the presence of a functioning PNET, medical control of the patient's symptoms prior to surgical intervention is of utmost importance. Although a detailed discussion of these treatments is beyond the scope of this review, this generally entails strict regulation of blood glucose levels in insulinomas, proton pump inhibitor treatment in gastrinomas, *etc*. In addition, a multidisciplinary approach involving the endocrinologist, surgeon, and anesthesiologist is essential in order to ensure safe resection.

TECHNICAL ASPECTS OF LAPAROSCOPIC SURGERY FOR PNETS

Various surgical techniques for laparoscopic pancreatic surgery have been described in the literature with some modifications that are based on surgeons' experience and preferences[13,22,48,53,55,59]. The following descriptions outline the important aspects and steps that are the basis for laparoscopic resections of PNETs. It is to be noted that the procedural description of laparoscopic pancreaticoduodenectomy (Whipple Operation) will not be described in this review.

Enucleation of tumors of the pancreatic head
After appropriate exposure of the pancreas, intraoperative laparoscopic ultrasonography is performed. Due to
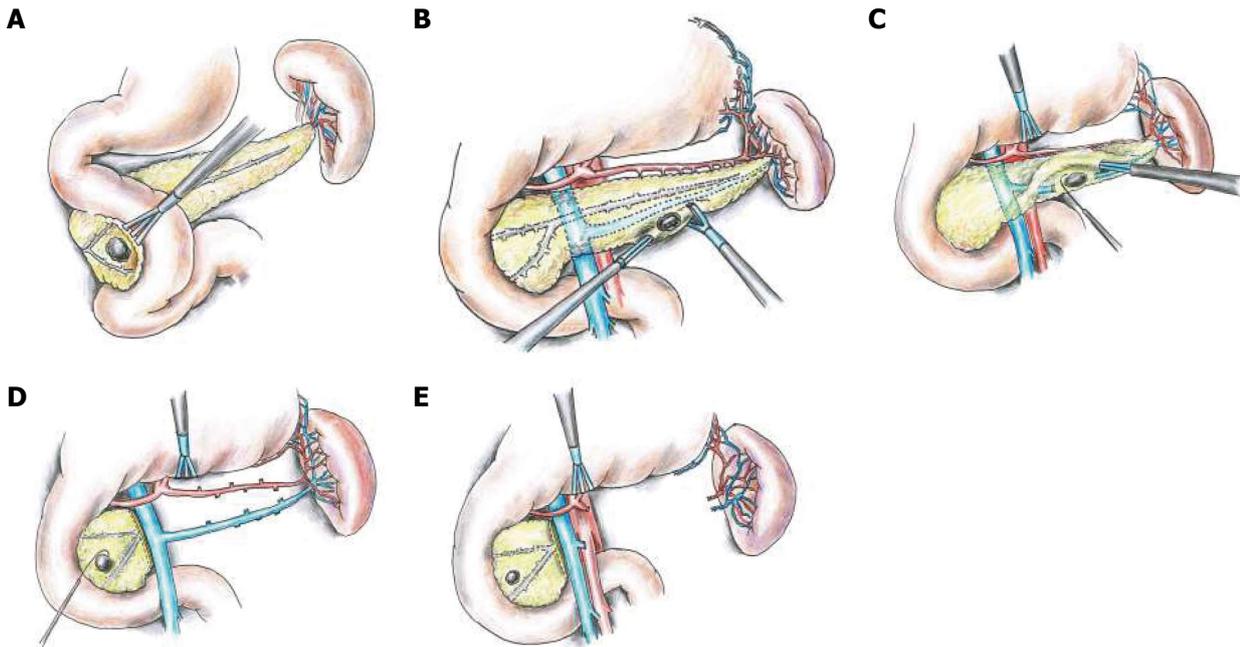
Figure 1 Tumors of the pancreatic head. A: A pancreatic neuroendocrine tumor (PNET) involving the posterior aspect of the pancreatic head, after adequate exposure by extensive kocherization and medial retraction of the pancreatic head, prior to enucleation; B: A PNET involving the inferior border of the body/tail of the pancreas. Resection is being performed using the LigaSure device; C: PNETs located in the posterior aspect of the body/tail occasionally require partial resection of the splenic vein in order to perform successful enucleation; D: The intraoperative appearance after performance of spleen-preserving distal pancreatectomy with splenic vessel preservation; E: The intraoperative appearance after performance of spleen-preserving distal pancreatectomy without splenic vessel preservation. Figure 1D and E represent patients with multiple endocrine neoplasia-1, with an additional PNET located in the head. This synchronous tumor will be excised by enucleation.

Figure 2 An image from intraoperative ultrasound demonstrating an insulinoma involving the pancreatic tail.

the lack of tactile sensation in laparoscopic surgery, this imaging tool is of utmost importance. It helps localize the tumor, rules out the presence of multiple lesions, and identifies the tumor's relation to and distance from surrounding structures[60]. Depending on the location of the tumor, focal dissection is continued in order to provide maximal exposure prior to enucleation (Figure 1A shows a PNET involving the posterior aspect of the pancreatic head, following appropriate exposure). Laparoscopic ultrasound is used to again identify the exact location of the tumor and its relation to the Wirsung duct and the superior mesenteric vein (SMV). Under extensive care not to damage these structures (which would lead to postoperative leak in the case of damage of the pancreatic duct),

electrocautery with the hook coagulator is utilized to dissect the parenchyma surrounding the tumor and perform enucleation. In tumors located at the inferior surface of the pancreatic head, the LigaSure device (Tyco, United States Surgical Volleylab, Boulder, Co. United States) may be used to incise the plane between the pancreas and the tumor. A surgical drain is left at the excision bed[48].

Enucleation of tumors of the pancreatic body and tail
After sufficient surgical exposure and mobilization of the pancreatic body and tail, laparoscopic ultrasonography is performed for tumor localization and to identify the relationship with surrounding structures (the pancreatic duct and splenic vessels). Figure 2 demonstrates the intraoperative sonographic appearance of an insulinoma involving the pancreatic tail.

When the tumor is located anteriorly, an incision is made in the pancreatic capsule using electrocautery, and delicate dissection is carried out between the tumor and the normal pancreatic parenchyma until successful enucleation of the mass is achieved. Bleeding is controlled by clips and cautery.

Tumors located at the inferior pancreatic border are commonly resected by hemostatic dissection using the LigaSure device (Figure 1B).

Posteriorly-located tumors are commonly partially covered by the splenic vein. The inferior border of the pancreas is lifted up, allowing exposure of the posterior pancreatic surface. Occasionally enucleation is only possible after local resection of the adjacent portion of the

vein (Figure 1C). In this process, injury to the splenic artery must be avoided. After enucleation, the tumor bed must be examined for evidence of pancreatic duct injury.

Tumors located in the distal portion of the tail of the pancreas are in very close proximity to the Wirsung Duct, and therefore enucleation of these tumors is commonly not recommended[48].

Spleen-preserving distal pancreatectomy with splenic vessel preservation

Exposure and mobilization of the body and tail of the pancreas is performed, as is mobilization of the splenic flexure. Adhesions are divided between the posterior surface of the stomach and the pancreas; however, care should be taken not to divide the short gastric and the left gastroepiploic vessels. After detaching the inferior pancreatic margin from the retroperitoneum, visualization of the posterior aspect of the pancreas is now feasible, as is identification of the SMV and the splenic vein forming the portal vein. Blunt dissection around the splenic vein is performed, with ligation of the small bridging vessels that reach the pancreas. After identification and preservation of the splenic artery, the pancreas is divided using an endoscopic stapler device. The body/tail of the pancreas is then anteriorly retracted, allowing further separation of small bridging vessels reaching the pancreas from the splenic artery and vein. The resection is completed after reaching the splenic hilum, and a surgical drain is left in proximity to the pancreatic stump[48] (Figure 1D).

Spleen-preserving distal pancreatectomy without splenic vessel preservation

This procedure follows the same course mentioned above until visualization of the posterior aspect of the pancreas and the splenic vein entering the SMV to form the portal vein. At this stage, clips are applied to the splenic vein and it is divided. The pancreas is then divided by endoscopic stapler, followed by ligation and division of the splenic artery. The pancreatic body and tail are retracted upwards (along with the attached splenic artery and vein), and these vessels are clipped and divided between the pancreatic tail and the splenic hilum. After this procedure, the sole remaining blood supply to the spleen is from the short gastric vessels and left gastroepiploic vessels, indicating the importance of their preservation in earlier steps[48] (Figure 1E).

Distal pancreatectomy with splenectomy

This procedure is similar to the previous technique (Spleen-preserving distal pancreatectomy without splenic vessel preservation) with a few exceptions. Unlike in spleen-preserving procedures, the short gastric and left gastroepiploic vessels can be ligated. In addition to mobilizing the splenic flexure (thereby exposing the inferior splenic border), the spleen's lateral aspect is also mobilized, up to the left crus of the diaphragm. The splenic vessels can be divided along with the pancreas or separately. The specimens are typically extracted from the

abdomen in two separate specimen bags. As in the previous procedure, a surgical drain is left *in situ*[13]. Note that some surgeons use different methods to seal the bed of tumor enucleation or the margins of resection, including adhesive biologic materials or sutures.

SURGERY IN PANCREATIC NEUROENDOCRINE CARCINOMA

According to the WHO 2010 classification, neuroendocrine carcinomas (NECs) are defined histopathologically as neuroendocrine tumors with a Ki-67 index above 20%[45]. These tumors are extremely invasive and aggressive, and fortunately they are rare, accounting for only 2%-3% of PNETs[45,61,62]. Radical surgery is generally indicated for locally advanced disease, followed by adjuvant chemotherapy. When there is evidence or suspicion of malignant disease, or when the tumor size is greater than 5 cm, it is recommended that a modified strasberg operation be performed[24]. This entails radical lymph node dissection of the peri-pancreatic, portal, hepatic, and superior mesenteric areas.

Not only does the literature support surgical excision of locally invasive disease, but a survival benefit has also been demonstrated after excision of metastases (in addition to the primary tumor) when technically feasible. Therefore, in selected patients with localized liver metastasis, it is recommended that a synchronous resection of the primary tumor and liver metastases should be attempted[14-18,63-68]. Nevertheless, the role of laparoscopy for these complicated procedures is yet to be clarified. Despite case reports of successful laparoscopic synchronous excision of the primary tumor and metastases this issue remains controversial as opponents claim that laparoscopic surgery may jeopardize the oncologic outcome and a planned open procedure must be carried out. As randomized controlled studies are unlikely this controversy will remain a matter of debate and it is reasonable to limit these procedures to highly experienced laparoscopic pancreatic/hepatobiliary surgeons.

SURGERY IN PNETS ASSOCIATED WITH MEN-1

More than 75% of patients with PNETs and MEN-1 have multiple pancreatic tumors, therefore enucleation alone in this clinical setting is likely to be inadequate[24,48]. Generally accepted indications for surgery in MEN-1 include the presence of a functioning PNET, in addition to nonfunctioning tumors of more than 2 cm in size[9,69]. However, some authors consider the diameter of 1 cm to be a safer cutoff, and advocate the surgical resection of nonfunctioning PNETs of more than 1 cm in diameter[69,70]. Smaller nonfunctioning tumors must be closely observed, and their rate of growth may subsequently provide an indication for surgical resection. The recommended surgical procedure for these patients seems to be

intraoperative laparoscopic ultrasonography, followed by subtotal distal pancreatectomy (usually with splenic preservation), along with enucleation of any lesions in the pancreatic head (Figures 1D and E).

OUTCOMES OF LAPAROSCOPIC PANCREATIC SURGERY FOR PNETS

As previously mentioned, surgery is the curative modality of choice for PNETs, improving survival across all stages of the disease. Recent years have shown a significant increase in the laparoscopic approach in these surgeries[18]. In several centers worldwide, almost all patients with suspicious PNET of the pancreatic body or tail undergo laparoscopic surgery[24,48,71,72].

In a recently published series, 75 laparoscopic procedures for PNETs were documented, of which 65 pancreatic resections or enucleations were performed[47]. The most common operation performed was distal pancreatectomy with splenectomy (n = 28), and this was followed by distal pancreatectomy without splenectomy (n = 23). The status of splenic vessel preservation was not clarified. Enucleation of a PNET of the head was performed in 7 cases, and of the body or tail in another 7 patients. The most common surgical complication was found to be post-operative pancreatic fistula (POPF), occurring in 21% of patients. This complication was more common in patients undergoing enucleation (50%) a finding that has been repeatedly shown in the literature, with a reported incidence of 13%-50% of POPF following enucleation[23,73-75]. Other "non-fistula" surgical complications had an incidence of 21%, and no perioperative mortality was demonstrated. In this study, a 5-year disease-specific survival of 90% was demonstrated, which can be compared to another series of 125 patients who underwent open surgical treatment of PNET and were found to have a 5-year survival of 65%[76]. However, issues of selection bias in these two different retrospective studies must be considered. DiNorcia *et al*[18] published a retrospective series in which 45 laparoscopic procedures for PNETs were compared to 85 open surgeries that were performed at the same institution. The two groups were similar with respect to gender, age, and race; however, as expected, a statistically significant difference was observed with regard to pathological characteristics of the tumors, with the laparoscopically operated group having smaller, lower-grade tumors, with less local and lymph node invasion. This study showed no statistically significant difference in overall morbidity rate between the two groups (48.9% *vs* 57.6%, P = 0.34, laparoscopic *vs* open operations, respectively); however, major complications were more prevalent in the open surgery group (11.1% *vs* 28.2%, P = 0.03). No perioperative mortality was seen in the laparoscopic group, while in patients who underwent open surgery, the perioperative mortality rate was 3.5% (P = 0.55). Median length of hospital stay was found to be significantly shorter in the laparoscopy group (6 d *vs* 9 d). Within the 25.4 mo follow-up period of the laparo-

scopic group, a 4.4% recurrence rate was demonstrated, compared to a 15.3% recurrence after a median follow-up of 42.7 mo in the open surgery group. In the study by Fernández-Cruz *et al*[24] which included 49 patients undergoing laparoscopic surgery for PNETs, a higher perioperative complication rate among patients undergoing laparoscopic enucleation compared to those who underwent laparoscopic distal pancreatectomy (42.8% *vs* 22%, respectively, P < 0.001) was observed[24]. The main complication was POPF which also occurred more frequently in the enucleation group (38% *vs* 8.7%, P < 0.001). However, all fistulas following enucleation were successfully managed conservatively. No perioperative mortality was demonstrated.

Assalia *et al*[13] reported their experience with 17 cases of laparoscopically treated PNETs, and demonstrated a perioperative complication rate of 23%, and a POPF rate of 15.3%. No mortality or recurrence was shown, although their series did not include patients with malignant neuroendocrine tumors. In the same publication, a review of an additional 93 reported cases of laparoscopically managed PNETs from the literature was also presented. These cases demonstrated a perioperative complication rate of 28%, and a POPF rate of 17.9%. Following enucleation, the fistula rate was higher than that following distal pancreatectomy (30.7% *vs* 5.1%, respectively). Fistulas were managed mainly by drainage alone (11/14), with a combination of drainage and ERCP with stenting (1/14), and two cases required reoperation. No mortality was observed.

In the literature, the reported rate of "conversion to open" in laparoscopic pancreatic surgery ranges from 8%-33%[24,77-82]. Reasons for conversion include intraoperative complications such as bleeding, inability to localize the tumor, or location of the tumor in close proximity to vital structures (such as the main pancreatic duct or portal vein) in which continuation of laparoscopic resection would either jeopardize those structures or would prevent an appropriate oncologic resection. However, as previously mentioned, the presence or suspicion of a malignant lesion in itself is not an indication for conversion or open surgery. A multi-center study compared open distal pancreatectomy with laparoscopic distal pancreatectomy for adenocarcinoma, and similar short- and long-term oncologic outcomes were demonstrated between the two groups[83]. Unfortunately, similar studies are not yet available for pancreatic neuroendocrine carcinomas.

CONCLUSION

Laparoscopy is a safe modality for the surgical treatment of PNETs. Retrospective studies demonstrated similar overall complication rates in comparison with open pancreatic surgery for these tumors; however, there is evidence that the rate of major complications is higher in those undergoing open surgery. Laparoscopy, although considered to be more technically demanding, is not associated with a compromise in terms of oncologic

outcome, and provides the benefits of decreased postoperative pain, better cosmetic results, shorter hospital stay, and a shorter postoperative recovery period. Further prospective, multi-center, and randomized trials are required for the analysis of these minimally invasive surgical techniques for the treatment of PNETs and their comparison to traditional open pancreatic surgery.

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Intervention on toll-like receptors in pancreatic cancer

Juan Vaz, Roland Andersson

Juan Vaz, Roland Andersson, Department of Surgery, Clinical Sciences Lund, Skåne University Hospital Lund, Lund University, SE-221 85 Lund, Sweden
Author contributions: Vaz J collected the underlying material and wrote the initial draft of the manuscript; Andersson R and Vaz J designed the outline of the paper; Andersson R finalized and revised the manuscript; both authors have read and approved this final version.
Correspondence to: Roland Andersson, MD, PhD, Department of Surgery, Clinical Sciences Lund, Skåne University Hospital Lund, Lund University, SE-221 85 Lund, Sweden. roland.andersson@med.lu.se
Telephone: +46-461-72359   Fax: +46-461-72335

Abstract

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease with pronounced morbidity and a high mortality rate. Currently available treatments lack convincing cost-efficiency determinations and are in most cases not associated with relevant success rate. Experimental stimulation of the immune system in murine PDA models has revealed some promising results. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to several forms of malignancy, including lung, breast and colon cancer. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas. In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as possible therapeutic targets. Most data points at TLR2 and TLR9 as effective tumor markers and agonists could potentially be used as *e.g.* future adjuvant therapies. The elucidation of the role of TLR3 in PDA is only in its initial phase. The inhibition/blockage of TLR4-related pathways has shown some promising effects, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Finally, TLR7 antagonists seem to be potential candidates for therapy. Independent of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology and development of PDA.

Core tip: The combination of high mortality rates and a tremendously complex pathophysiology makes pancreatic ductal adenocarcinoma (PDA) an enormous challenge. We summarize the current knowledge about the importance of toll-like receptors (TLR) in PDA. Since both tumor and tumor-related cells express TLRs, intervention on TLR-related pathways may represent future candidates for therapy.

INTRODUCTION

Disorders of the pancreas are leading causes of morbidity and mortality. Despite advanced surgical and/or oncological treatment strategies, pancreatic ductal adenocarcinoma (PDA) is still associated with an extremely poor prognosis with a median survival of 6 mo and a 5-year survival rate less than 1%-2%[1,2]. PDA represents the fourth cause of cancer-related deaths and its incidence is rising in most countries[3].

The causes of PDA are mainly unknown. A family history is found in up to 5%-10% of patients[4]. Known

risk factors for PDA are among others tobacco smoking, diabetes mellitus, obesity and chronic pancreatitis[5-7]. Pancreatic intraepithelial neoplasia (PanIN) in the ductal epithelium has been suggested as the primordial precursor of PDA[8]. As PanIN progress to carcinoma, accumulated mutations might result in the activation of the *KRAS2* oncogene, loss of CDKN2A/p16 and/or the inactivation of TP53 and SMAD4[9]. Likewise, stellate cells are major players in PDA, as they are fundamental for the development of the characteristic desmoplastic stroma found in PDA[10]. Pancreatic cancer stem cells might be important in treatment resistance and metastasis. A large range of cell populations, such as tumor-associated macrophages (TAMs), have been reported as central in PDA[11,12]. The current knowledge of the pathophysiology of PDA has elegantly been summarized by Hidalgo[13,14].

At the time of diagnosis, most patients have already developed locally advanced (stages Ⅱ or Ⅲ) or metastatic (stage Ⅳ) disease and palliative treatment is the only alternative. Gemcitabine is a nucleoside analogue with a broad-spectrum against solid tumors that for long has been used as first-line treatment. In PDA, gemcitabine increases the quality of life of many patients, but merely prolongs the mean survival by one month[15]. Furthermore, a majority of patients do not respond to gemcitabine due to lack of the necessary nucleoside transporter, and the total costs and side-effects related to gemcitabine overtreatment are high[16,17]. FOLFIRINOX (5FU/leucovorin, irinotecan and oxaliplatin) is currently a first-line treatment for metastatic PDA as the regime is more active than gemcitabine at overall survival, progression-free survival and response rate. Moreover, the degradation of the quality of life is also delayed by FOLFIRINOX[18]. However, the regime is more expensive than gemcitabine and not suitable for all patients due to its toxicity. Hence, in most developing countries, gemcitabine is still the gold standard. Thus, current chemotherapeutic strategies lack proper cost-efficiency determinations and are not effective in the vast majority of cases.

In order to increase survival rates in PDA, it is imperative to find novel therapies that specifically target tumor cells and/or associated cell populations and stroma. Toll-like receptors (TLRs) are pillars of the immune system that have been linked to major cancer forms, including lung, breast and colon cancer[19-21]. In humans, TLRs are expressed in the pancreatic cancer tissue and in several cancer cell lines, whereas they are not expressed in the normal pancreas[22,23] (Table 1). TLRs thus appear to play a role in the pathophysiology of PDA (Table 2, Figure 1) and may thereby also represent targets for intervention (Table 3). In the present review, we explore the current knowledge concerning the role of different TLRs associated to PDA.

TOLL-LIKE RECEPTORS

TLRs are pattern recognition receptors that recognize numerous pathogen-associated molecular patterns (PAMPs) derived from virus, pathogenic bacteria, pathogenic fungus and parasitic protozoa. Likewise, TLRs can start immunological reactions against endogenous molecules released into the extracellular compartment under *e.g.*, stress or tissue damage[24]. TLRs are type Ⅰ integral membrane glycoproteins expressed in various cell compartments, and in humans the expression of ten different TLRs (TLR1 to TLR10) has been reported[25]. Upon activation, TLRs form heterodimers or homodimers, and an activating signal is started. After the recruitment of adaptor molecules, TLRs can activate two major intracellular signaling pathways. All TLRs, except TLR3, can activate a MyD88-dependent pathway, causing the transcription of pro-inflammatory genes through the activation of nuclear factor κβ (NFκB) and/or the activation of activating protein 1[24,26]. An alternative, non MyD88-dependent pathway, can be initiated by TLR3 and TLR4. In the TRIF-pathway, the activation of interferon-regulated factors (IRF) *via* TRIF results in the synthesis of interferon (IFN) and/or the activation of NFκB[24].

TLR2-PROMISING ADJUVANT THERAPY

Mainly expressed on the plasma membrane, TLR2 is found in a large diversity of cells of the immune system[27]. In addition to its role in infectious diseases, TLR2 has been associated to *e.g.*, atherosclerosis, asthma and renal disease[28-30].

Macrophage activating lipopeptide-2 (MALP-2) is a synthetic lipopeptide that activates immune responses through TLR2 and TLR6[31,32]. In syngeneic subcutaneous and in orthotopic murine models the local administration of MALP-2 results in significant tumor growth reduction and prolonged survival[33]. Furthermore, the MALP-2 anti-tumor effect is enhanced by co-treatment with gemcitabine. However, the metastatic potential of cancer cells is not reduced by MALP-2 administration. MALP-2 might exert its effects through CD8+ lymphocytes and NK-cells since the murine Panc-2 cell line used for this experiment do not express TLR2. Hypothetically, MALP-2 activates dendritic cells (DCs) in a TLR2/TLR6-dependent manner[34]. A subsequent phase Ⅰ/Ⅱ trial showed promising results[35]. Ten patients in different PDA disease stages were included, both with "radical" surgery or palliative procedures leaving the pancreatic tumor behind. MALP-2 was injected intratumorally during surgery and six patients received adjuvant chemotherapy. The drug was well tolerated and a mean survival of 17.1 mo was observed. The median survival was 9.3 mo and no metastases were reported during follow-up. Despite the limited number of patients, the reported mean survival was remarkably high. The local administration of MALP-2 appears to upregulate the activation of both the innate and the adaptive immune system, resulting in decreased tumor proliferation and metastasis. Still, it is unclear if MALP-2 has a future as adjuvant therapy in PDA, since no further trials have been reported up to date. In addition, several less expensive TLR2 agonists appear to have similar biochemical properties when compared to

Table 1 Toll-like receptors found in human pancreatic adenocarcinoma cell lines

Cell line	Source	Phenotype	Expressed TLR	Ref.
AsPC-1	Metastasis: ascites	Du	TLR3, TLR4, TLR9	[23,57,58]
BxPC-3	Primary tumor	Du	TLR2-4	[40,50,57]
CFPAC	Metastasis: liver	Du	TLR4	[57]
Colo357	Metastasis: lymph node	Un	TLR3, TLR7	[48]
GER	Primary tumor	An	TLR9	[72]
MIA PaCa-2	Primary tumor	An	TLR2-4, TLR7, TLR9	[23,40]
MDAPanc-28	Primary tumor	Du/Ac	TLR2-4, TLR7, TLR9	[23]
Panc-1	Primary tumor	Du/An	TLR2-4, TLR7, TLR9	[23,40,50,58,79]
Panc-89	Metastasis: lymph node	Du	TLR3 , TLR7	[48]
PancTu-1	Primary tumor	Du	TLR3, TLR7	[48]
Pt45P1	Primary tumor	Du	TLR3, TLR7	[48]
SU.8686	Metastasis: liver	Du	TLR2	[41]
SW-1990	Metastasis: spleen	Du	TLR2-4, TLR7, TLR9	[23]
T3M4	Metastasis: lymph node	Du	TLR9	[77]

TLR: Toll-like receptor; Du: Ductal; Ac: Acinar; An: Anaplastic; Un: Undefined.

Table 2 Toll-like receptors expressed in pancreatic ductal adenocarcinoma and their reported implications

	Pathophysiological significance	Ref.
TLR2	Cell growth	[33,40,43]
	Immunosuppression	[33,41,43]
	Mean survival	[33,35]
	Progression and metastasis	[43]
TLR3	Carcinogenesis	[47]
	Cell growth and migration	[50]
	Immune responses	[48]
TLR4	Angiogenesis	[63]
	Carcinogenesis	[49]
	Cell growth	[49,57,61]
	Epithelial-to-mesenchymal transition	[61]
	Leukocyte recruitment and genomic instability	[57]
	Mean survival	[62]
	Progression and metastasis	[49,58,61]
	Stromal expansion	[49,61]
TLR7	Carcinogenesis, stromal expansion, progression and metastasis	[67]
	Immune responses	[48]
TLR9	Cell growth	[77,79]
	Mean survival	[77]
	Metastasis	[72,77,79]

TLR: Toll-like receptor.

MALP-2.

Protein-bound polysaccharide-K (PSK, Krestin®) is a natural remedy derived from highly purified mushroom extracts (*Trametes versicolor*) that since decades has been used as adjuvant therapy in cancer[36]. Even if the mechanisms are only partially known, PSK is thought to be a novel TLR2 agonist and it has documented therapeutic effects in colorectal and lung cancer[37-39]. Moreover, PSK promotes apoptosis and inhibit tumor growth in various human PDA (hPDA) cell lines[40]. Even if PSK-related cancer cell apoptosis is unlikely to be mediated through TLR2, the inhibition of the later significantly reduced the positive effects of PSK in all cell lines challenged. Thus, TLR2-pathways might be (if only in part) involved in the tumor suppressor effect of PSK.

TLR2 is also a promising cell-surface target since its protein expression is specifically increased in hPDA tissue[22]. Designed, fully synthetic high affinity TLR2 agonists have been studied with encouraging outcome. Derived from natural TLR2 ligands and also from MALP-2, these new compounds are able to induce the immune system when given as vaccine adjuvants in murine PDA (mPDA) models[41]. These results imply a potential in developing high affinity tumor targeted therapies through TLR2. A particularly potent compound has been conjugated with a near-infrared fluorescent dye, the novel Dmt-Tic-Cy5. The combination of Dmt-Tic-Cy5 and 3D imaging methods was applied in the intraoperative detec-

tion of tumor masses in a mouse xenograft model[42]. Using Dmt-Tic-Cy5 as a tumor marker during surgery in mice, successful R0 resections were obtained. Future applications of this technic could include the detection of early tumors or the improvement of current surgical procedures in hPDA.

Pancreatic adenocarcinoma upregulated factor (PAUF) is a protein overexpressed in hPDA and other types of cancer[43]. PAUF appears to modulate the metastatic potential of cancer cells and it upregulates the expression of CXCR4, the later being related to increased cancer cell motility[44]. PAUF induce the expression of the cytokines RANTES and MIF *via* TLR2 and it is also associated with the inhibition of CXCR4-dependent and TLR2-mediated NFκB activation, with subsequent decreased tumor necrosis factor-α levels[45]. Theoretically, PAUF might contribute to tumor persistence *via* the disruption of TLR2-dependent anti-tumor pathways in cancer.

In summary, TLR2 is not only expressed in tumor tissue but also in several hPDA cell lines (Table 1). Since TLR2 is present in both primary tumor cell lines and in cell lines from metastases, the receptor may be a novel target for immunotherapy in hPDA. The clinical significance of TLR2-targeting can become important in the future since the marker is present in up to 70% of resected tumors[22] but mainly absent in the normal pancreas. While the pathophysiological role of TLR2 in mPDA seems to be complex (Table 2, Figure 1), TLR2 agonists have shown promising results in animal models and in a phase I / II clinical trial (Table 3).

TLR3-UNEXPLORED IMPLICATIONS

TLR3 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of DCs and mono-
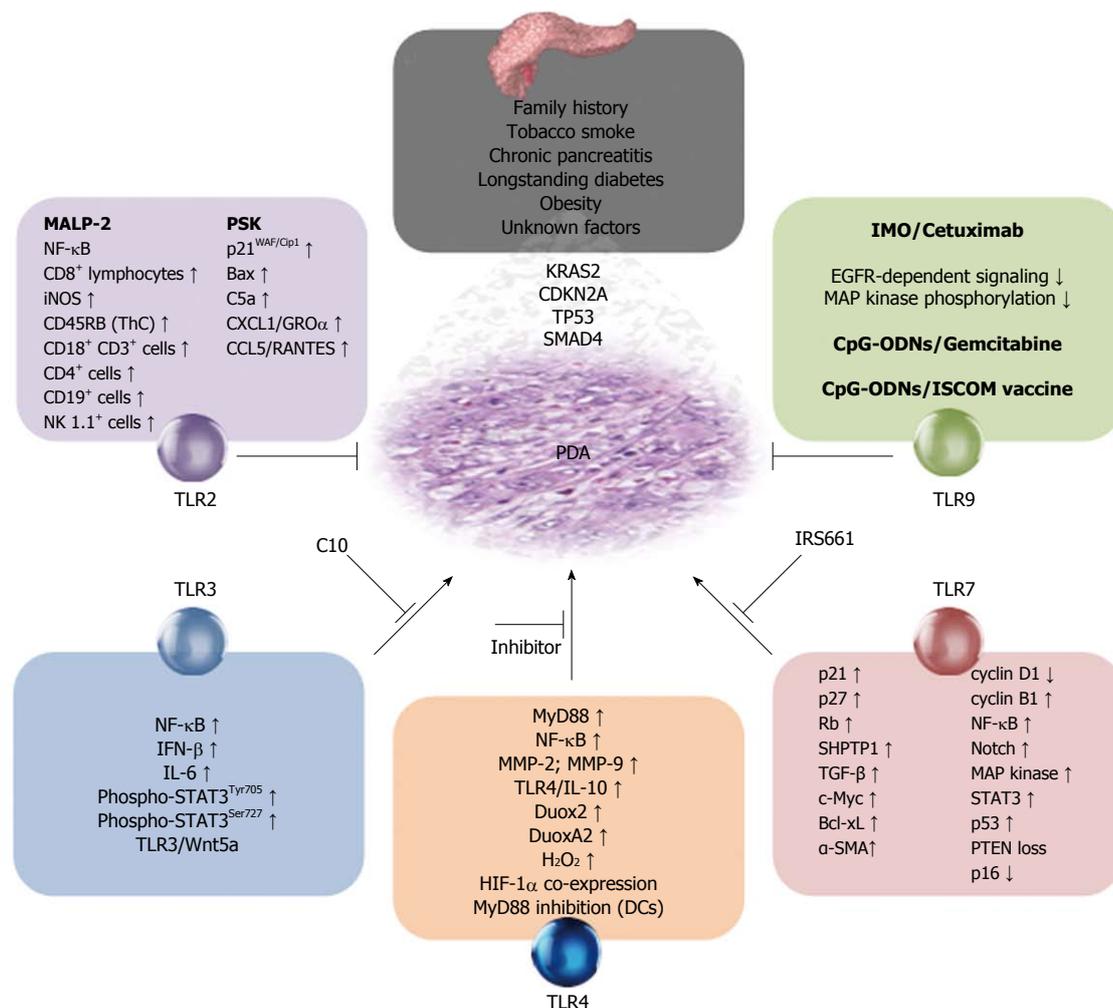
Figure 1 Toll-like receptors in the pathophysiology pancreatic ductal adenocarcinoma. TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PSK: Polysaccharide-K; C10: Phenylmethimazole; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides; TGF-β: Transforming growth factor-β; α-SMA: α-smooth-muscle antibody. NF-κB: The nuclear factor NF-κB.

cytes. Besides its role in viral infections, TLR3 has been linked to chronic pancreatitis and breast cancer[46,47].

Polycytidylic acid (Poly I:C) is a well-known TLR3 agonist capable of inducing cell lysis in hPDA cell lines by enhancing the cytotoxic activity of γδ T cells *in vitro*[48]. However, Poly I:C has also been reported to accelerate pancreatic carcinogenesis in KRAS-mutated mice[49].

TLR3 expression in hPDA cell lines is correlated with increased tumor cell growth and constitutive Wnt5a expression[50]. Wnt-associated pathways are related to a vast variety of cellular processes in embryogenesis and carcinogenesis[51]. Phenylmethimazole (C10) is a TLR3 inhibitor able to suppress the dsRNA induced, TLR3-mediated IRF3/IFN-pathway, independent of Wnt5a. The administration of C10 leads to less tumor development in a xenograft murine model. Importantly, C10 decreased TLR3 expression and significantly inhibited hPDA cell growth and motility/migration. The expression of TLR3 in tumor cells might result in increased interleukin (IL)-6 levels[52]. C10 effects could then be mediated by the inhibition of phosphorylated STAT3 *via* the disruption of TLR3/Wnt5a-related pro-inflammatory IL-6 expression

in hPDA.

Even if TLR3 is constitutively expressed in primary hPDA cell lines (Table 1), it is unclear which role TLR3 plays in hPDA. Opposite results have been reported when TLR3 inhibitors have been tried. Hence, no conclusions can be made at this point.

TLR4-IS INHIBITION THE ANSWER?

Being the first TLR identified, TLR4 is widely expressed as homodimers or heterodimers with TLR6 on the plasma membranes of many immune cells. TLR4 has been linked to several diseases, including obesity, acute pancreatitis and breast cancer[18,53,54].

TLR4 is overexpressed both in mPDA and hPDA[49]. Stromal leukocytes from patients have increased TLR4 expression. Interestingly, the upregulation is also found both in epithelial and stromal cells in KRAS-mutated mice. Moreover, TLR4-inhibition in these mice had protective effects against tumorigenesis and TLR4^{-/-} animals had a slower tumor growth. However, the inhibition of MyD88-dependent and TRIF-pathways had opposite

Table 3 Toll-like receptors and their intervention in pancreatic ductal adenocarcinoma

	Substance/compound	Intervention	Effects	Ref.
TLR2	MALP-2 (G)	Activation	Induce lymphocyte invasion and tumor necrosis	[33]
			Inhibit tumor growth	[33]
			Prolongs mean survival	[35]
			Reverse tumor-associated immunosuppression	[33]
	Polysaccharide-K (G)	Activation	Inhibit tumor growth and induce apoptosis in tumor cells	[40]
	Dmt-Tic-Cy5	Activation	Acts as vaccine adjuvant in pancreatic cancer	[41]
			Target imaging and therapy	[41,42]
	PAUF	Mixed	Facilitates tumor growth	[43]
			Promotes tumor immune-resistance	[43]
TLR3	Polycytidylic acid	Activation	Accelerates carcinogenesis	[49]
			Induces T cell invasion and tumor lysis	[48]
	Phenylmethimazole	Inhibition	Inhibits tumor growth and migration	[50]
TLR4	Lipopolysaccharide	Activation	Accelerates carcinogenesis	[49]
			Induce desmoplastic stroma	[49]
			Induce increased H_2O_2 extracellular production	[57]
			Increased invasiveness	[58,61]
			Induce M2-polarization in tumor-associated macrophages	[61]
TLR7	Imiquimod	Activation	Induce T cell invasion and tumor lysis	[48]
	IRS661	Inhibition	Prevent tumor progression and stromal expansion	[67]
			Regulates cell cycle in cancer cells	[67]
TLR9	CpG-ODN 1816/26 (G')	Activation	Delays tumor development, reduce invasiveness	[72]
			Prolongs mean survival	[72]
	IMO (C)	Activation	Prolongs mean survival, inhibit tumor growth and migration	[77]
			Reestablish cetuximab sensibility in cancer cells	[77]
	CpG-ODN 2216	Activation	Inhibits tumor growth and migration	[79]

TLR: Toll-like receptor; MALP-2: Macrophage activating lipopeptide-2; PAUF: Pancreatic adenocarcinoma upregulated factor; IRS661: Immunoregulatory sequence 661; CpG-ODN: CpG oligodeoxynucleotide; IMO: Immunomodulatory nucleotides. (G): Synergism when combined with gemcitabine; (G'): Effect mainly when combined with gemcitabine; (C): Effect merely when combined with cetuximab.

effects in mPDA. While MyD88-inhibition clearly accelerated tumor development and gave rise to highly aggressive TP53 mutated cancer cells, TRIF-inhibition had antitumor effects. MyD88-inhibition could induce aggressive cancer cells even in TRIF-deficiency co-existence.

Even if MyD88 blockage has been associated with a decreased tumor development in other cancer forms[55], the presence of DCs in PDA microenvironment appears to be the main factor for MyD88-dependent tumor-stimulating effects. Upon MyD88 blockage, DCs seem to induce pancreatic antigen-restricted Th2-deviated CD4+ T cells[49]. Furthermore, the abundance of Th2 cells in hPDA is linked to a worsened prognosis[56].

Inflammatory cytokines can induce NFκB activation in mPDA. LPS and INF-γ challenge results in increased production of extracellular H_2O_2 in primary hPDA cell lines[57]. Through TLR4, the activation of NFκB might enhance the transcription of dual oxidase 2, trigger leukocyte recruitment and genetic instability. In hPDA cell lines, LPS challenge induced improved invasiveness *via* TLR4/MyD88-depending pathways[58]. Moreover, RNAi silencing TLR4 or MyD88 completely reversed the effects of LPS. NFκB activation might induce increased expression of matrix metalloproteinases (MMPs) in mPDA. MMP-2 and MMP-9 overexpression is related to the progression of hPDA, and its blockage has been subject of intensive research[59]. Thus, LPS may act through a TLR4-MyD88-NFκB axis that finally leads to MMP-9 overexpression and thereby to increased invasiveness *in vitro*[60].

The overexpression of MMPs has also been coupled to TAMs. M2-polarized TAMs mediate EMT, induce cancer cell proliferation and migration in hPDA cells *in vitro*[61]. These effects may partially be achieved through TLR4. TLR4 overexpression in M2-polarized macrophages could lead to IL-10 release with impact on the EMT and thereby on the metastatic potential of the cancer cells.

hPDA is characterized by a poor vascularization. Thus, the role of angiogenesis in hPDA remains controversial[9]. In humans, hypoxia-inducible transcription factor alpha (HIF-α) is overexpressed in resected pancreatic cancer tissue. Moreover, a positive correlation between mRNA/protein HIF-α levels and mRNA/protein TLR4 levels in primary tumors and metastases has been found. TLR4 was expressed in 69.2% of the analyzed tumor tissue. Besides, the expression of either TLR4 or HIF-α was related to a decreased survival rate and when both were expressed, an accumulative effect was observed[62]. Some data imply that hypoxia in solid tumors, such as hPDA, induces HIF-α overexpression, which might be responsible for the expression of TLR4 in hPDA cells *in vitro* and in a xenograft murine model[63]. Here, TLR4 was found in 76 % of the tumor tissue but no data on average survival or prognosis was presented.

The inhibition/blockage of TLR4-related pathways has shown some promising results, but there are still many steps left before TLR4 inhibitors can be considered as possible therapeutic agents. Since both stromal cells and primary tumor cells express TLR4, it is plausible

that TLR4 ligands found in the inflammatory tumor microenvironment initiate complex interactions between the different cell populations. This might in turn lead to the secretion of tumor stimulating cytokines and the recruitment of further cell populations into the tumor stroma. Since hypoxia and TLR4 ligands are common in the tumor stroma, the upregulation of TLR4 and HIF-α in hPDA could be auto-stimulatory. Poor prognosis can then be partially predicted, as a highly hypoxic tumor stroma is less sensitive for radiotherapy and disrupt the delivery of chemical agents into the primary cancer cells.

TLR7-PROMOTING CANCER PROGRESS

TLR7 is a nucleic acid-recognizing receptor expressed as dimers on endosomal membranes of APCs and leukocytes. TLR7 activation is currently used for the treatment of various malignancies, such as melanoma and breast cancer[64]. Like TLR3, TLR7 has also been used to enhance cytotoxic activity in γδ T cells *in vitro*[48].

The role of TLR7 in mPDA has been reported previously[65,66]. Upregulated TLR7 is found in epithelial cells and macrophages, DCs, neutrophils, and B- and T-cells of the tumor microenvironment. In hPDA, the expression of TLR7 is increased both in epithelial ductal cells and inflammatory cells within the tumor stroma.

Moreover, the administration of ssRNA40, a TLR7 agonist, results in pronounced tumor growth and stromal expansion in mice. In KRAS-mutated mice, the tumor-stimulating effects of TLR7 appears to be mediated by a complex array of events, including loss of expression of PTEN, p16 and cyclin D1 and upregulation of among others p21, p27, p53, c-Myc, SHPTP1, TGF-β, PPARγ and cyclin B1. Moreover, ssRNA40 challenge resulted in the activation of STAT3, MAP kinase, Notch and NFκB pathways. Notch target genes were downregulated, giving rise to the hypothesis that Notch, together with NFκB, might mediate inflammation in the tumor microenvironment, thus promoting tumor persistence and metastatic potential[67].

Importantly, TLR7 stimulation is not self-sufficient for malignant transition when KRAS mutations are absent. Equally important, mice with TLR7-/- phenotype seem to be protected against tumor progression. The administration of IRS661, an oligonucleotide inhibitor of TLR7, prevented tumor progression and stromal expansion in mice[67]. IRS661 treatment decreased the expression of p21, p27, p-p27, cyclin B1, CDK4 and p-STAT3 in mice with invasive PDA. Thus, TLR7 inhibition was able to affect cell cycle regulation in already formed pancreatic tumors. However, the expression of Rb or TP53 was not affected by IRS661.

The evidence of the importance of TLR7 in mPDA is strong and TLR7 antagonists are without doubt promising experimental adjuvant agents that must be further evaluated. Importantly, PanIN in humans do not express TLR7 with the same intensity as established hPDA tumors. Moreover, the expression of TLR7 appears to

increase with tumor progression and it is found in nearly 50% of the advanced tumors[67]. TLR7 may induce tumor progression in a KRAS-dependent manner since the mutation must be present for TLR7-mediated tumor progression in mice. As KRAS2 is mutated in over 90% of hPDA[18], these may only be a minor obstacle for the future clinical use of TLR7-targeting.

TLR9-AGONISTS AS FUTURE ADJUVANT THERAPY?

As TLR3 and TLR7, TLR9 is expressed on endosomal membranes of several immune cells, including macrophages, B cells and DCs[68]. Besides its role in bacterial, viral or malaria infection, TLR9 has been linked to acute pancreatitis and cancer[54,69].

Synthetic TLR9 agonists (CpG-ODNs) are oligodeoxynucleotides containing CpG motifs that have been used as vaccine adjuvants or as antiallergic agents[70]. In combination with vaccines based on immune stimulatory complexes, a TLR9 agonist inhibits the tumor immune evasion in mPDA[71]. It is believed that CpG-ODNs can activate NK-cells, DCs and cytotoxic T cells, thus initiating anti-tumor immune responses. TLR9 is highly expressed in the tumor microenvironment and in circulating leukocytes in a murine xenograft PDA model. CpG-ODNs treated mice had a reduced tumor spread to the diaphragm, liver and spleen and the combination of gemcitabine and CpG-ODNs resulted in delayed development of bulky disease, less metastasis and improved survival, when compared to gemcitabine monotherapy[72].

The epidermal growth factor receptor (EGFR) is overexpressed in 50%-60% of hPDA[73]. Cetuximab is a monoclonal anti-EGFR antibody that has shown promising results experimentally, but not clinically in hPDA[74,75]. Immunomodulatory nucleotides (IMO) are second-generation CpG-ODNs with higher metabolic stability. IMO interferes with EGFR-dependent signaling and has thereby a synergistic effect with anti-EGFR agents[76]. In combination with cetuximab, IMO inhibits cell growth in hPDA and cancer progression in KRAS-mutated murine cell lines[77]. Importantly, in cetuximab-resistant cell lines, IMO potentiated the activity of cetuximab. The administration of IMO resulted in tumor growth inhibition and prolonged survival in a murine xenograft model. The associations between EGFR/TLRs interactions and carcinogenesis are slowly being elucidated. However, the impact on hPDA is still unexplored[78].

Another CpG-ODN (ODN2216) has shown antiproliferative properties in an hPDA cell[79]. Tumor cell growth, replication rate and migration ability were decreased in cells challenged with ODN2216. The effects seem to be time- and dose-dependent. Moreover, the expression of TLR9 is more pronounced in hPDA tissue than in peritumoral ones (73.3% *vs* 33.3%)[79].

As TLR2, TLR9 appears to be a promising tumor marker. Likewise, TLR9 agonists could be used as adjuvant therapy by themselves or in combination with al-

ready established chemotherapies (Table 3). Nonetheless, the pathophysiological role of TLR9 in hPDA is mainly unexplored.

CONCLUSION

The role of the immune system in cancer is an area of intensive research. Cancer cells have the ability to evade immune responses and promote tumor phenotypes and pathways in immune cells. TLRs are related to several cancer forms, and immunotherapies involving TLRs are a reality[27]. At least thirty new clinical trials evaluating TLRs agonist and cancer have started since May 2012[80].

The combination of high mortality rates and a tremendously complex pathophysiology makes PDA an enormous challenge. The role of inflammation and immune cells in PDA cannot be stressed enough[81]. Both MyD88-dependent cascades and TRIF-pathways have been associated with tumor growth, survival and metastatic potential in PDA[65]. Even if almost all known TLRs are expressed in the pancreatic cancer microenvironment, there are only five TLRs suggested as potential therapeutic targets.

Importantly, the effects of TLRs agonists and antagonists in PDA are presumably mediated by the inducement of anti-tumor immune response. This requires access to the primary tumor site. Moreover, TLR-targeting can theoretically disrupt important pathways in primary tumor cells with therapeutic effects. Thus, TLR-based agents must either be administered intratumorally or delivered through the tumor stroma. The recognition of specific tumor targets is then imperative for the application of TLRs intervention in PDA. In clinical practice, CA 19-9 is widely used as hPDA marker. CA 19-9 is a relatively specific marker useful as indicator for advanced disease or tumor recurrence after surgery. However, as pancreatic cancer progress and spreads beyond the pancreas, the accumulation of abnormalities might change the sensitivity and/or specificity of tumor markers since metastases may differ profoundly from the primary tumor[82]. We have recently propose mucin 4 (MUC4) as a novel tumor marker in hPDA. MUC4 is found in both primary and matched metastatic tumors with a high level of concordance (82 %)[83]. Specific tumor markers open the door for efficient drug delivery *via e.g.*, nanotechnology. For instance, targeted liposomal delivery of TLR9 ligands in cancer has already been evaluated with encouraging results[84].

Independently of their potential in immunotherapies, all existing data indicate that TLRs are strongly involved in the pathophysiology of PDA (Figure 1). The role of TLRs in PDA is not limited to the direct effect on tumors or associated cells. TLRs are also involved in the pathophysiology of several risk factors for hPDA, such as chronic pancreatitis, diabetes and obesity[47,85].

The present paper summarizes the current understanding of interventions on TLRs in PDA. Despite initial encouraging results, further research and elucidation of involved mechanisms is demanded.

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2014 ADVANCES IN PANCREATIC CANCER

Endoscopic ultrasound in the diagnosis of pancreatic intraductal papillary mucinous neoplasms

Alkiviadis Efthymiou, Thrasyvoulos Podas, Emmanouil Zacharakis

Alkiviadis Efthymiou, Thrasyvoulos Podas, Department of Gastroenterology, St Luke's Hospital, Thessaloniki 55236, Greece

Emmanouil Zacharakis, Department of Surgery, St Luke's Hospital, Thessaloniki 55236, Greece

Alkiviadis Efthymiou, Department of Gastroenterology, Bioclinic Hospital, Thessaloniki 54622, Greece

Author contributions: Efthymiou A conceptualized and designed the manuscript; Efthymiou A and Zacharakis E reviewed the literature; Efthymiou A drafted the manuscript; Efthymiou A, Zacharakis E and Podas T revised and finally approved the manuscript.

Correspondence to: Alkiviadis Efthymiou, MD, PhD, FEBG, Department of Gastroenterology, St Luke's Hospital, Kliniki "agios Loukas", Thessaloniki 55236,
Greece. a_efthimiou@hotmail.com
Telephone: +30-2310-234623 Fax: +30-2310-234633
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Abstract

Pancreatic cystic lesions are increasingly recognised due to the widespread use of different imaging modalities. Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas represent a common, but also heterogeneous group of cystic tumors with a significant malignant potential. These neoplasms must be differentiated from other cystic tumors and properly classified into their different types, main-duct IPMNs *vs* branch-duct IPMNs. These types have a different malignant potential and therefore, different treatment strategies need to be implemented. Endoscopic ultrasound (EUS) offers the highest resolution of the pancreas and can aid in the differential diagnosis, classification and differentiation between benign and malignant tumors. The addition of EUS fine-needle aspiration can supply further information by obtaining fluid for cytology, measurement of tumor markers and perhaps DNA analy-sis. Novel techniques, such as the use of contrast and sophisticated equipment, like intraductal probes can provide information regarding malignant features and extent of these neoplasms. Thus, EUS is a valuable tool in the diagnosis and appropriate management of these tumors.

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Key words: Endoscopic ultrasound; Pancreatic intraductal papillary mucinous neoplasms

Core tip: This review shows that endoscopic ultrasound initially provides differential diagnosis of pancreatic cystic tumors and subsequently can classify intraductal papillary mucinous neoplasms of the pancreas into their different types. With the use of endoscopic ultrasound (EUS) fine-needle aspiration and other techniques, such as contrast enhancement, EUS can differentiate between benign and malignant neoplasms and help the clinician to implement the proper treatment strategy.

INTRODUCTION

Intraductal papillary mucinous neoplasms of the pancreas (IPMNs) are a well-recognised disease entity since their first report by Ohashi *et al*[1] in 1982. They consist of pancreatic tumors characterised by papillary proliferation of the ductal epithelium which produces mucin and is accompanied by dilatation of the excretory pancreatic

ducts. World Health Organization formally differentiated IPMNs from other mucin-producing cystic lesions of the pancreas in 1996, through a uniform classification scheme[2]. IPMNs have been reported with increased frequency, representing 21%-41% of all cystic neoplasms of the pancreas[3,4]. The increased detection of pancreatic cystic lesions, including IPMNs, is due to the widespread use of various abdominal imaging modalities, such as ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI)[5].

IPMNs represent a heterogeneous group of neoplasms that are classified according to their anatomic location into main duct *vs* branch duct IPMNs. There is also a mixed-type IPMN which is the combination of the above two types. IPMNs involving the main duct (MD) have a higher risk of associated carcinoma, compared to branch duct (BD) IPMNs[6]. IPMNs also comprise a wide histologic spectrum that ranges from adenoma to invasive carcinoma with different degrees of aggressiveness[7]. Early detection and precise anatomical and histological classification are therefore of paramount importance for the optimal management of these tumors.

Despite advances in CT and MRI, the ability of cross-sectional imaging modalities to characterize pancreatic IPMNs correctly, and to differentiate between benign and malignant lesions remains limited. Endoscopic ultrasound (EUS) is an ideal diagnostic tool for the imaging of pancreatic cystic lesions and therefore IPMNs, because of its high resolution, close proximity to the target-lesion and ability to take samples, by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA)[8].

This review focuses on the true prevalence of IPMNs, the role of EUS in the detection, differential diagnosis and classification of these neoplasms but also the impact of EUS/EUS FNA on the ultimate management of these tumors.

CLINICAL AND EPIDEMIOLOGICAL CHARACTERISTICS OF IPMNS

IPMNs account for 1%-3% of all exocrine pancreatic neoplasms and for 21%-41% of all cystic neoplasms of the pancreas[9-11]. The exact incidence of IPMNs, however, is not known because many of them are small and asymptomatic. Imaging studies revealed that asymptomatic cysts of the pancreas that presumably contain predominantly small IPMNs were found in 2.8% of 2832 consecutive CT scans performed in a single institution, and this figure rose to 8.7% in individuals aged > 80 years[12]. IPMNs are typically found in elderly people, with a median age at diagnosis of 65-70 years. As stated earlier, IPMNs may involve the main pancreatic duct (MD-IPMN), in either a diffuse or segmental manner, or may arise in a branch duct (BD-IPMN). Any combination of the above two types is designated as mixed-type IPMN. Most IPMNs arise from the main duct within the head or the uncinate process of the pancreas and progress along the duct[13]. BD-IPMNs are characterised by the presence

of multifocal cystic lesions in different sites of the gland, sometimes with a complete involvement of the entire pancreas.

Microscopically, the epithelium is represented by tall, mucin-producing columnar cells that frequently form papillary projections and exhibit variable degrees of cellular atypia, even within an individual neoplasm. Non-invasive IPMN is graded according to the most atypical area as IPMN with low-grade dysplasia (adenoma), IPMN with moderate dysplasia (borderline), and IPMN with high-grade dysplasia (carcinoma *in situ*). If an invasive component is present, which occurs in 30%-50% of cases, the tumor is called an IPMN with an associated invasive carcinoma[9,14-16]. Progression from adenoma to carcinoma is estimated to occur within 5-6 years[15,16]. The frequency of malignancy (in situ and invasive carcinoma) in MD-IPMNs is high, ranging between 60% and 92%, with a mean of 70%[6,15-19]. By contrast, in BD-IPMNs the frequency of malignancy is significantly lower (between 6% and 46%, with a mean of 25%) and that of invasive cancer ranges from 0% to 30% (mean of 15%)[6,15,17,19-21].

Another unique feature of IPMNs is their association with malignancy in other organs. Breast, colorectal, lung and prostate cancers are the most common extra-pancreatic tumors. The rate of association of IPMNs with malignant neoplasms in extrapancreatic organs has been reported to range from 23.6% to 32%[22,23].

EUS for the detection of IPMNs
IPMNs occur most frequently in men during the age of 60-70 years, but they can sometimes concern younger patients[13,24]. Detection of IPMNs is usually done by other imaging modalities rather than EUS itself. The usual clinical scenario is that of a cyst found by abdominal ultrasound, CT, or MRI, either incidentally or during diagnostic work-up in patients presenting with abdominal pain, recurrent acute pancreatitis, or chronic obstructive pancreatitis. Pain and pancreatitis are both caused by mucin-induced obstruction of the pancreatic duct[24]. The reported prevalence of pancreatic cystic lesions is 1.2%-2.9%, when CT is used[12,25], and 13.5%-44.7%, when MRI is the imaging modality[26,27].

EUS is a more invasive diagnostic procedure which allows high-resolution imaging of the pancreas. Since the vast majority of patients will have undergone prior abdominal imaging before presenting for EUS, the latter is carried out as part of a multi-modality diagnostic evaluation[28].

DIFFERENTIATION BETWEEN IPMNS AND OTHER PANCREATIC CYSTIC LESIONS BY EUS MORPHOLOGY

Cystic pancreatic lesions include pseudocysts, congenital and simple cysts and cystic neoplasms. Cystic neoplasms consist of serous cystadenoma (SCA), mucinous cystic neoplasm (MCN) and IPMN. In addition, there are

Figure 1 Multilocular branch-duct intraductal papillary mucinous neoplasms in the uncinate process of the pancreas (communication with the pancreatic duct is just visible).

Figure 2 Branch-duct intraductal papillary mucinous neoplasms communicating with a non-dilated pancreatic duct.

pancreatic tumors that contain cystic spaces or cystic degeneration components, like solid-pseudopapillary neoplasm, cystic endocrine tumor and ductal adenocarcinoma). Certain morphological features have been used to predict particular types of pancreatic cysts. A cyst with accompanying features of pancreatitis, in the absence of septations and mural nodules, suggests a pseudocyst[29]. Multiple microcysts (< 3 mm) within a cystic lesion, occasionally with a honeycomb-like appearance in an asymptomatic patient strongly suggests serous cystadenoma. On the other hand, MCNs are usually cysts with septations of variable thickness, a visible wall and peripheral calcifications in up to 15% of cases[30]. IPMNs are usually macrocystic-type lesions, occasionally accompanied by parenchymal changes due to obstruction of the duct, which communicate with the pancreatic duct (Figure 1). If this communication can be identified by EUS, it definitely distinguishes IPMNs from MCNs or macrocystic SCAs, which both do not communicate with the ductal system[29].

Classification into MD-IPMN and BD-IPMN by EUS

The identification of a connection between the cystic lesion and the pancreatic ductal system is suggestive of the diagnosis of IPMN. This is usually feasible with the use of EUS, which due to its high resolution and proximity of the transducer to the pancreas, gives an excellent imaging of the ductal system.

EUS findings in IPMNs include segmental or diffuse, moderate to marked dilatation of the main pancreatic duct, often associated with intraductal nodules. Dilatation of the main pancreatic duct $\geqslant$ 1 cm strongly suggests MD-IPMN. However, according to "the international consensus guidelines 2012 for the mamagement of IPMN and mucinous cystic neoplasms of the pancreas", the threshold of main duct dilatation has been lowered to > 5 mm without other causes of obstruction[31]. This change has increased the sensitivity for diagnosis of MD-IMPN without losing specificity.

The presence of a cyst communicating with the pancreatic duct without main duct dilatation or with main duct diameter < 6 mm suggests BD-IPMN (Figure 2).

The finding of multiple cysts supports the diagnosis of BD-IPMN, as these tumors are more frequently multifocal than MD-IPMNs. In mixed-type IPMNs, in addition to the presence of BD-IPMN, the main pancreatic duct contains papillary growth of columnar epithelium of various degrees of dysplasia. Pancreatic parenchymal atrophy is also frequently recognised in both MD-IPMNs and BD-IPMNs. Since, MD-IPMNs and BD-IPMNs have significant differences in prevalence of cancer, the correct classification has prognostic implications[24,31].

Differentiation between benign and malignant pancreatic IPMNs

Invasive carcinoma has been reported in 33%-60% of cases in larger series of resected MD-IPMNs[16,19,32,33]. A pancreatic main duct > 1 cm in diameter, the presence of mural nodules and/or symptoms (especially jaundice and diabetes) are risk factors of invasive cancer[15,16,18,34]. A similar incidence of invasive cancer has been reported in main duct and mixed-type IPMN[34]. Hence, mixed-type IPMN should be considered as a main duct disease. Based on the high prevalence of malignancy, all patients fit for surgery with MD- or mixed type-IPMN should undergo resection[28].

BD-IPMNs harbor invasive carcinoma in 11%-30% of cases in large series[19,21,32,35]. The presence of mural nodules, dilatation of the main pancreatic duct > 6 mm, a growth rate over 2 mm/year, the presence of symptoms and elevated serum levels of CA 19-9 are all considered to be risk factors and indications for resection[36-42]. Cyst size greater than 3 cm has been shown to be associated with malignancy in a few studies[17]. However, later studies showed that cyst size alone was not a predictive factor of malignancy, since cancer was found in smaller lesions[43,44]. Furthermore, it was reported that even cysts larger than 3cm can be followed safely, as long as there are no other signs of malignancy[45]. Thus, dimension correlates with the risk of cancer, but there is no safe lower size limit that completely excludes malignancy[28].

The above data imply that EUS can differentiate benign from malignant IPMNs by accurately measuring the size of the cyst, the diameter of the main pancreatic duct and by detecting the presence of mural nodules.
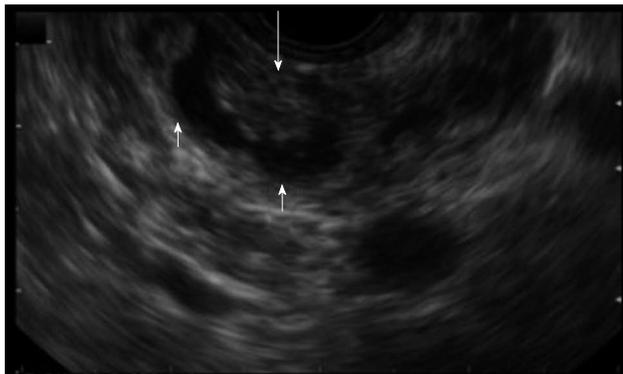
Figure 3 Main-duct - intraductal papillary mucinous neoplasms with a mural nodule. This figure shows a dilated main pancreatic duct (short arrow), with a mural nodule within the duct (long arrow). This patient was found to have a main-duct - intraductal papillary mucinous neoplasms with an invasive adenocarcinoma.

In a study done to investigate the value of EUS in differentiating malignant from benign IPMN, 51 patients with IPMN were preoperatively examined by EUS. The histopathological findings of the resected specimens were compared with the endosonographic findings. MD-IPMN with MD dilatation ≥ 10 mm, BD-IPMN (greater than 40 mm) with irregular septa, and large mural nodules (greater than 10 mm) strongly suggested malignancy on EUS[46].

The presence of mural nodules in patients with IPMNs was shown to be a strong predictor of malignancy and may help determine whether the treatment strategy should be surgical resection or conservative management[20,21,45,47,48]. EUS is the most sensitive imaging modality used to detect mural nodules[29,49-51] (Figure 3). Most BD-IPMNs without mural nodules on EUS remained unchanged during long-term follow-up, suggesting that BD-IPMNs without mural nodules could be managed conservatively[37,45]. A study from Baba *et al*[50], comparing EUS with ultrasound, CT and magnetic resinance cholangiopancreatography (MRCP) concluded that EUS was the most effective in differentiating between benign IPMNs from malignant tumors, by assessing the height of protrusion of lesion within the cysts. In a study from Hara *et al*[52], using intraductal ultrasonography, 88% of lesions protruding 4mm or more were malignant. A recent study from Kim *et al*[53], showed that BD-IPMNs smaller than 16 mm, without main pancreatic duct dilatation can be safely followed-up with CT or MRCP, while BD-IPMNs greater than 16 mm or with main pancreatic duct dilatation need an initial EUS evaluation for detection of mural nodules. Recently, Kobayashi *et al*[54] studied 36 patients with BD-IPMN and found that the diameter of the mural nodule of papillary protrusions and the width diameter reliably distinguished low-risk from high-risk IPMNs (4.3 mm *vs* 16.4 mm and 5.7 mm *vs* 23.2 mm, respectively).

Role of contrast-enhanced EUS in IPMNs

Contrast-enhanced harmonic EUS is often used to examine the microvasculature and perfusion in the pancreas.

Contrast-enhanced EUS (CE-EUS) detects signals from microbubbles produced by intravenously administered contrast agents and filters signals originating from tissues by selectively detecting harmonic components. This technology can detect signals from microbubbles in vessels with very slow flow without Doppler-related artifacts and is used to characterize vascularity[55]. Different second-generation ultrasound contrast agents, designed and optimized with regard to their resistance to pressure, have been developed for CE-EUS. They consist of microbubbles which are filled with different chemicals, *e.g.*, sulfur hexafluoride (Sonovue, Bracco United Kingdom Ltd., United Kingdom) or galactose (Levovist, Nihon Schering Co., Ltd., Tokyo, Japan), *etc*. After intravenous injection of these agents, vascularity is temporarily enhanced, allowing for better morphological evaluation of lesions[51,55,56]. Kitano *et al*[55] reported that contrast-enhanced endoscopic sonography was a useful tool for characterising pancreatic tumors. Using contrast-enhanced EUS, Ohno *et al*[51] classified mural nodules of IPMN into four types: type Ⅰ: low papillary nodule, type Ⅱ: polypoid nodule, type Ⅲ: papillary nodule, type Ⅳ: invasive nodule. The diagnosis of IPMNs with type Ⅲ or Ⅳ mural nodule had a sensitivity of 60%, specificity of 92.9% and accuracy of 75.9% for predicting malignancy. Same authors, by using contrast-enhanced EUS, also showed that the existence of mural nodules and involvement of the main pancreatic duct at initial presentation of patients with BD-IPMNs were significant predictors of malignant transformation[56].

It is sometimes difficult to precisely evaluate the presence of mural nodules, as they can not be easily distinguised from mucous clots. Contrast-enhanced EUS discriminates mural nodules from mucous clots in IPMNs by evaluating the vascularity of the protrusions: nodules are vascular, whereas clots are not. A recent study showed that the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of contrast-enhanced EUS for mural nodule detection were 100%, 80%, 92%, 100% and 94%, respectively[57].

ROLE OF EUS-FNA

EUS has the advantage of allowing real-time guidance of FNA of cystic lesions. This is very important considering that EUS morphology alone has a diagnostic accuracy of just 50% in patients with pancreatic cysts[3]. The fluid obtained by EUS-FNA is mainly used for cytology and measurement of tumor markers. Cytological specimens in IPMNs may be characterized by abudant mucin, very little inflammatory cells, neoplastic cells either single, cohesive or forming papillae and mucinous epithelium[58]. Immunostaining can demonstrate cells positive for mucin 1, mucin 2 or mucin 5AC[7] (Figure 4).

The most important differential diagnosis achieved by EUS-FNA is the distinction between mucinous (including IPMNs) and non-mucinous cysts. A recent meta-analysis demonstrated that EUS with cyst fluid cytology could differentiate between mucinous and non-mucinous lesions

Figure 4 Fine-needle aspiration of a branch-duct intraductal papillary mucinous neoplasms. Material obtained was mucinous (Papanikolaou staining) with low cellularity. Mucinous cells did not demonstrate nuclear atypia and expressed MUC5AC, but not MUC1 or MUC2 on immunostaining, findings consistent with a benign branch-duct intraductal papillary mucinous neoplasms.

with a sensitivity of 54% and specificity of 93%[59], while fluid's CEA sensitivity was 63% and specificity 88%[59].

A cut-off of ≥ 192-200 ng/mL is approximately 80% accurate for the diagnosis of a mucinous cyst[31,60,61]. However, a low CEA level does not exclude a mucinous cyst. Cyst fluid amylase is not uniformly elevated in IPMN, and MCN may also exhibit elevated amylase levels[61]. Serous cysts typically have low levels of both CEA and amylase, while pseudocysts have amylase levels > 250 U/L, but low CEA levels[28]. In our experience it is difficult to differentiate between IPMNs and pseudocysts by cyst fluid markers alone, since there is an overlap between values of amylase and CEA levels in these two entities.

Fernández-Esparrach *et al*[62], demonstrated that EUS-FNA had a sensitivity of 82%, a specificity of 100%, positive predictive value of 100%, negative predictive value of 92% and accuracy of 94% in diagnosing IPMNs. Although cytology can be helpful in the diagnosis of IPMNs, its sensitivity is limited in many studies by the scant cellularity of the specimen[60,63,64]. In a recent study from Lim *et al*[65], 132 patients with cystic pancreatic lesions underwent EUS-FNA. Pseudocysts and IPMNs were the predominant lesions in the cohort and cytologic yield was 47%. However, when a solid component was present in the cyst, doing more than one pass increased the diagnostic yield from 44% to 78%.

Cyst fluid analysis can also help in identifying malignancy in IPMNs. In a study from Pais *et al*[66], the sensitivity, specificity and accuracy of EUS-FNA for the diagnosis of malignancy in IPMNs were 75%, 91%, and 86%, respectively, while the level of CEA was of limited value. Similarly, a recent study from Kucera *et al*[67], showed that CEA level of cyst fluid is a poor predictor of malignancy within an IPMN. The 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 was 52.4%, 42.3%, 42.3%, 52.4% and 46.8%, respectively, in the above study.

In cases of small sample size, DNA analysis may be possible and looks promising[68]. Recently, the presence of K-ras mutation was found helpful in the diagnosis of mucinous cysts with a specificity of 96%[69]. Detection of K-ras mutations supports a mucinous rather than malignant cyst. Recent studies indicated that Guanine nucleotide binding protein (G protein), alpha stimulating activity polypeptide 1 mutations (GNAS mutations) may be helpful in distinguishing mucinous cysts from indolent cysts that can be managed conservatively. GNAS mutations of codon 201 which are unique to IPMNs have been detected in more than 60% of IPMNs[70,71]. DNA analysis, therefore accurately identifies mucinous cysts, including IPMNs.

In summary, EUS-FNA has its limitations in identifying IPMNs, but by combining cytology, measurement of tumor markers and perhaps DNA analysis where possible, it can definitely aid in the diagnosis of these tumors. Cytology and measurement of CEA must always be performed in the fluid of a pancreatic cyst, while DNA analysis is still experimental and not widely available. Cystic fluid analysis alone is not usually adequate for identifying IPMNs. It's results should always be interpreted in conjunction with clinical information and EUS morhologic findings.

INTRADUCTAL ULTRASONOGRAPHY

Intraductal ultrasonography (IDUS) has higher resolution than EUS due to the higher ultrasound frequency. The probes that are used have a small size (5-10 Fr) and their scanning frequencies are between 12 and 30 MHz. After being inserted into the duct, either by free cannulation or over a guidewire, during standard ERCP, these probes give an image of the pancreatic duct with one or three layers[72]. IDUS has been reported as a reliable tool for a more detailed evaluation of pancreatic tumors, especially IPMNs[73,74]. Hara *et al*[52] reported that the combination of peroral pancreatoscopy and IDUS resulted in a considerably improved differential diagnosis between malignant and benign IPMN. Yamao *et al*[75] reported that the combination of EUS and intraductal ultrasonography showed great accuracy in the diagnosis of invasive IPMN. IDUS was also found to be useful in preoperative localization and prediction of extension of IPMN[72]. Kobayashi *et al*[76] performed IDUS in 24 patients with BD-IPMN and detected lateral spread of these tumors in 54% of cases. In this group of patients, the main pancreatic duct had a diameter of more than 6 mm. However, there is an inverse relationship between high ultrasound frequency and depth of penetration, which means that these probes have limited utility in the detection of lesions more than a few millimetres away from the pancreatic duct[24]. Preoperative IDUS may therefore be beneficial for the determination of resection line in IPMNs.

In summary, IDUS is helpful in differentiating malignant and invasive IPMNs from benign ones and also in determining the extent of surgical resection. However, it is a more invasive and less available procedure than standard EUS which can only be performed in tertiary centres and not in routine clinical practice.

FUTURE EUS DEVELOPMENTS

Existing tumor markers in the cyst fluid have limited value in the diagnosis of IPMNs and more sensitive biomarkers need to be identified. Proteomics and molecular analysis are new techniques that may be helpful in the differential diagnosis of pancreatic cysts and thus, the identification of IPMNs[77].

In vivo real-time imaging can be performed using confocal laser endomicroscopy. This thechnology involves the EUS-guided placement of a miniprobe through a 22G needle inside lesions located near the digestive tract[78]. It is possible that EUS-guided confocal laser endomicroscopy might be practised soon and possibly aid in the diagnosis of IPMNs by giving *in vivo* histologic images of the pancreatic cysts.

Surgical resection is the treatment of choice when IPMNs meet the criteria mentioned earlier. However, recent studies showed that pancreatic cyst ablation with ethanol or ethanol followed by paclitaxel is feasible[79-81]. Studies included patients with IPMNs, but they generally had a short follow-up of patients for documentaion of cyst resolution. Therefore, EUS-guided ablation is currently experimental and should be used only in patients who refuse surgery or are high-risk surgical candidates[82]. Further prospective studies with longer follow-up are necessary.

CONCLUSION

IPMNs represent an increasingly common diagnostic and therapeutic challenge. Once a pancreatic cyst is detected, usually by other imaging modalities, EUS will greatly help in the differential diagnosis. It can accurately classify IPMNs into MD-IPMNs, BD-IPMNs or mixed-type tumors and also identify features highly indicative of malignancy. The use of contrast can also point out malignant features, such as mural nodules, and EUS-FNA can give further information by providing fluid for cytology, tumor markers and possibly DNA analysis. IDUS, if available, is useful in the evaluation of IPMNs, allowing accurate localization and prediction of extension. Therefore, EUS is a valuable tool in the diagnosis and further management of these intriguing cystic tumors of the pancreas.

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Applications of endoscopic ultrasound in pancreatic cancer

Leticia Perondi Luz, Mohammad Ali Al-Haddad, Michael Sai Lai Sey, John M DeWitt

Leticia Perondi Luz, Mohammad Ali Al-Haddad, Michael Sai Lai Sey, John M DeWitt, Division of Gastroenterology and Hepatology, Indiana University, Medical Center, Roudebush VA Medical Center, Indianapolis, IN 46202, United States

Correspondence to: John M DeWitt, MD, Division of Gastroenterology and Hepatology, Indiana University, Medical Center, Roudebush VA Medical Center, 550 N University Blvd., UH 4100, Indianapolis, IN 46202, United States. jodewitt@iupui.edu
Telephone: +1-317-9441113   Fax: +1-317-9488144

Abstract

Since the introduction of endoscopic ultrasound guided fine-needle aspiration (EUS-FNA), EUS has assumed a growing role in the diagnosis and management of pancreatic ductal adenocarcinoma (PDAC). The objective of this review is to discuss the various applications of EUS and EUS-FNA in PDAC. Initially, its use for detection, diagnosis and staging will be described. EUS and EUS-FNA are highly accurate modalities for detection and diagnosis of PDAC, this high accuracy, however, is decreased in specific situations particularly in the presence of chronic pancreatitis. Novel techniques such as contrast-enhanced EUS, elastography and analysis of DNA markers such as k-ras mutation analysis in FNA samples are in progress and might improve the accuracy of EUS in the detection of PDAC in this setting and will be addressed. EUS and EUS-FNA have recently evolved from a diagnostic to a therapeutic technique in the management of PDAC. Significant developments in therapeutic EUS have occurred including advances in celiac plexus interventions with direct injection of ganglia and improved pain control, EUS-guided fiducial and brachytherapy seed placement, fine-needle injection of intra-tumoral agents and advances in EUS-guided biliary drainage. The future role of EUS and EUS in management of PDAC is still emerging.

Core tip: Applications of endoscopic ultrasound (EUS) in pancreatic cancer are emerging. We review the role of EUS in the detection, diagnosis and staging of pancreatic cancer. The introduction of recent novel techniques such as contrast-enhanced EUS, elastography and analysis of DNA markers in fine-needle aspiration samples might improve the accuracy of EUS. In addition, we review therapeutic application of EUS including celiac plexus interventions, fiducial and brachytherapy seeds placement, fine needle injection and EUS-guided biliary drainage.

INTRODUCTION

With the introduction of endoscopic ultrasound guided fine-needle aspiration of pancreatic masses by Vilmann *et al*[1] (EUS-FNA), endosonography has assumed an increasing role in the management of pancreatic ductal adenocarcinoma (PDAC). In this review, our objective is to discuss the various applications of EUS and EUS-FNA in PDAC. Initially, its use for detection, diagnosis and staging, including newly described techniques as

contrast-enhanced EUS, elastography, and use of DNA markers will be described. Finally, the use of therapeutic EUS procedures including celiac plexus neurolysis and emerging therapies as fine-needle injection, implantation of fiducials and brachytherapy seeds will be discussed.

DETECTION AND DIAGNOSIS OF PANCREATIC CANCER

Detection of pancreatic cancer

EUS is the most sensitive nonoperative imaging test for the detection of malignant pancreatic lesions, with a reported sensitivity between 87%-100%[2-11]. EUS is markedly superior to transabdominal ultrasound (reported sensitivity between 64%-91%)[2-5,7] and has also been shown to be superior to computed tomography (CT) (sensitivity 66%-86%) for the detection of pancreatic masses in studies which compared both techniques[3-8,10,11]. EUS is clearly superior to conventional CT[3-5,8] and a few studies comparing EUS and multidetector-row CT (MDCT) for detection of pancreatic tumors demonstrated the superiority of EUS as compared to 4-row CT[10,11]. Agarwal *et al*[10] showed a sensitivity of 100% for EUS in the diagnosis of cancer compared to 86% for MDCT in a retrospective cohort of 81 patients with PDAC. DeWitt *et al*[11] reported similar findings in a prospective cohort of 80 patients with PDAC, showing that the sensitivity of EUS 98% statistically superior to MDCT 86% for detection of PDAC. There are scant comparisons between EUS and MRI for tumor detection with at least one study showing superiority of EUS[6] and one study showing superiority of magnetic resonance imaging (MRI)[9]. Future studies comparing EUS and 3.0 or higher Tesla MRI are necessary to further define the roles of each imaging modality in the diagnosis of pancreatic masses.

EUS is particularly useful for identification of small tumors that are not visualized by other imaging modalities[3,6,10-12] For tumors ⩽ 30 mm in diameter, EUS was found to have a sensitivity of 93% compared to 53% for CT and 67% for MRI[6]. In a recent retrospective cohort by Wang *et al*[12], which included 116 patients with clinical presentation suspicious for PDAC and inconclusive MDCT findings, EUS showed a sensitivity of 87% and an accuracy of 92% in diagnosing pancreatic neoplasm. With thinner slice imaging and precisely timed contrast administration coupled with multiplanar reconstruction, pancreas protocol CT may now be able to identify small pancreatic masses that previously may have been undetected by conventional or even single detector dual-phase imaging[11]. EUS should be performed in all patients with obstructive jaundice or unexplained pancreatic and/or bile duct dilations in whom CT or MRI do not definitively identify a pancreatic lesion, both to detect any tumor and to exclude non-neoplastic diseases.

EUS may fail to identify a true pancreatic mass in patients with chronic pancreatitis, a diffusely infiltrating carcinoma, a prominent ventral/dorsal split or a recent episode (< 4 wk) of acute pancreatitis[13]. In a study of 80 patients with clinical suspicion of PDAC and a normal EUS, Catanzaro *et al*[14] found that no patient with a normal pancreatic EUS developed cancer during a follow-up period of 24 mo. Therefore, a normal pancreas by EUS examination essentially excludes PDAC although follow-up EUS or other studies should be done in the setting of chronic pancreatitis due to potentially impaired visualization. Acoustic shadowing caused by an indwelling biliary or pancreatic stent may also interfere with visualization of a small pancreatic mass. However, a recent retrospective study by Ranney *et al*[15] did not show any difference in the diagnostic yield or technical difficulty of EUS-FNA of visualized pancreatic masses in the presence of a biliary stent (plastic or metal).

Imaging-based technologies such as contrast-enhanced EUS (CE-EUS) may be used to differentiate PDAC from other benign or malignant lesions. In this procedure, an intravenous contrast agent is administered at time of EUS and microbubbles are detected in the microvasculature of pancreatic tumors during real-time evaluation. Adenocarcinomas show hypo-enhancement while neuroendocrine tumors and pseudotumoral chronic pancreatitis are iso- or hyper-enhancing. Numerous contrast agents are available including first generation contrast agents such as Levovist and second generation such as Sonovue and Sonazoid. In a recent meta-analysis including 1139 patients, the pooled sensitivity and specificity of CE-EUS for the differential diagnosis of pancreatic adenocarcinoma were 94% and 89%, respectively[16]. This study found that a hypoenhanced lesion by CE-EUS was a sensitive and accurate predictor of adenocarcinoma. In the United States, routine use of CE-EUS is limited by its high cost and the lack of both agent availability and expertise with this technique.

Another emerging technology used to differentiate benign from malignant masses is EUS elastography. This technology provides real-time evaluation of tissue stiffness and is based on the premise that there is less strain when hard tissues are compressed compared to soft tissues[17]. As malignant lesions are generally harder than normal adjacent tissue, measuring strain might aid classification of pancreatic masses. Results from 2 recent meta-analyses demonstrated a high pooled sensitivity of 95%-97% but a low pooled specificity of 67%-76%, respectively, for differential diagnosis of solid pancreatic masses[18,19]. Elastography might provide complementary information to EUS, potentially increasing the yield of EUS-FNA, and assist endosonographers to improve targeting of FNA[18]. Limitations of this technique include limited availability, difficulty controlling tissue compression by the endosonographer, presence of motion artifacts, and unclear stiffness cut-off values for pancreatic masses[19].

Elastography and contrast-enhanced imaging may be combined during the same procedure. Săftoiu *et al*[20] sequentially combined CE power Doppler with real-time elastography in 21 patients with chronic pancreatitis and 33 patients with PDAC undergoing EUS examination. The sensitivity, specificity, and accuracy of combined information provided by both tests to differentiate hypo-
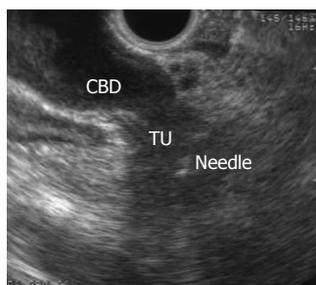
Figure 1 Endoscopic ultrasound guided fine-needle aspiration of pancreatic mass in a patient with painless jaundice.

vascular hard masses suggestive of pancreatic carcinoma were 75.8%, 95.2%, and 83.3%, respectively, with a positive predictive value and negative predictive value of 96.2% and 71.4%, respectively.

Diagnosis of pancreatic cancer

EUS-FNA of a pancreatic mass was first described in 1992[1] and is currently the preferred method to sample pancreatic mass lesions, having largely replaced intraoperative sampling or biopsies under CT or US guidance. EUS-FNA is performed using the linear array echoendoscope, as the ultrasound transducer at its distal tip allows needle advancement under real-time guidance once the target is identified (Figure 1). EUS-FNA of a suspected metastatic site from PDAC (ascites, distant metastatic lymph node, omental nodule or a suspicious liver lesion) should be performed first. If those are negative for malignancy then either the suspected tumor or a regional lymph node may be sampled.

EUS-FNA has excellent accuracy. Two recent meta-analyses reported a pooled sensitivity for the diagnosis of malignancy based on cytology of 85% and 89%, and a pooled specificity of 98% and 99%, respectively[21,22]. EUS-FNA of unresectable pancreatic cancer therefore is routinely performed where available but its use in patients with resectable pancreatic cancer remains controversial when neoadjuvant therapy is not planned.

EUS-FNA of pancreatic masses is overall a safe procedure. A recent systematic review by Wang *et al*[23] of 8246 patients with pancreatic lesions reported complications in 60 (0.82%) patients. Pancreatitis occurred in 36/8246 patients, of which 75% where mild. One patient with severe pancreatitis died, with an estimated pancreatitis-related mortality rate of 2.78%. The overall rate of pain, bleeding, fever and infection were 0.38%, 0.10%, 0.08% and 0.02% respectively.

Peritoneal seeding of tumor cells following EUS-FNA has been reported in up to 2.2% of patients but appears to be less than CT-guided FNA (16.3%)[24]. EUS-FNA did not increase the risk of peritoneal carcinomatosis in pancreatic masses in a comparison of 161 patients who underwent Endoscopic retrograde cholangiopancreatography (ERCP) alone with 56 who also underwent EUS-FNA[25]. Beane *et al*[26], compared overall and recurrence-free survival of patients with PDAC who underwent distal pancreatectomy, and found no differ-

ence between the 179 patients included who underwent preoperative EUS-FNA as compared with the 59 patients who did not. In addition, in a recent study, the risk of gastric/peritoneal recurrence after preoperative EUS-FNA was evaluated in 256 patients diagnosed with malignant pancreatic neoplasms who underwent surgery with curative intent, and it was found that EUS-FNA was not associated with increased needle track seeding[27].

Despite excellent accuracy and a low incidence of major complications, EUS-FNA of pancreatic masses has several limitations. Despite excellent sensitivity, negative predictive value of EUS-FNA for pancreatic tumor remains limited at 55%-65%[10,21]. Therefore, a negative or nondiagnostic FNA does not completely exclude the possibility of malignancy. Secondly, the presence of chronic pancreatitis decreases the diagnostic accuracy of EUS-FNA[28,29]. The presence of chronic pancreatitis may also hinder cytological interpretation of pancreatic FNA, decreasing sensitivity of EUS-FNA[30]. Third, EUS-FNA for pancreatic cancer has a false-positive rate of 1.1%, usually in patients with chronic pancreatitis[31].

The presence and experience of an on-site cytopathologist also impacts the accuracy of EUS-FNA[22,32] In a recent meta-analysis, which included 34 studies and 3644 patients, rapid on-site evaluation was a significant determinant of accuracy of EUS-FNA in the diagnosis of pancreatic masses[22]. The optimal number of EUS-FNA passes has been evaluated by 2 studies[32,33], which reported that at least 5-7 passes for pancreatic masses should be performed to maximize diagnostic yield. This information may prove helpful to endosonographers performing EUS-FNA when rapid pathology interpretation is unavailable.

A variety of commercially available FNA needles is available which range in size from 19 to 25 gauge (G). In a recent meta-analysis, 25-G needle was associated with a higher sensitivity but comparable specificity to the 22-G needle in 1292 patients with solid pancreatic lesions[34]. In another meta-analysis, 25-G needles appeared to have an advantage in adequacy of passes as compared to 22-G needles, without difference in accuracy, number of passes or complications[35]. Interestingly, 25-G needles were associated with less technical failures compared to 22-G needles when sampling pancreatic head and uncinate process lesions in some studies, and therefore should be considered first in those cases[36,37].

Due to its inherent rigidity, 19-G needles have been rarely used in the duodenum. Recently, a needle made of nitinol has been developed with enhanced flexibility to overcome these limitations (Flex 19, Boston Scientific, Natick, MA). The first report on the use of this needle included 38 patients, 32 of those with pancreatic head/uncinate lesions. Transduodenal FNA yielded adequate samples for cytological analysis in all 32 patients, without technical failures or procedure related complications[38].

EUS-FNA with use of DNA markers

In order to improve the diagnostic yield of EUS-FNA of pancreatic masses, analysis of abnormal genes in EUS-FNA samples is being investigated. The most studied

marker is κ-ras. A prospective study including 394 pancreatic masses found that the combination of κ-ras mutation analysis with cytopathology increased the sensitivity of EUS-FNA from 87% to 93% and the accuracy from 89 to 94%[39]. Recently, a meta-analysis of 8 prospective studies (931 patients) assessing the accuracy of k-ras mutation analysis in the diagnosis of PDAC reported a pooled sensitivity and specificity of 77% and 93%, respectively. When combined with EUS-FNA alone, the addition of k-ras mutation testing improved sensitivity from 81% to 89% but decreased specificity from 97% to 92% for the diagnosis of PDAC. Among inconclusive EUS-FNA cases, k-ras mutation analysis reduced the false-negative rate by 56% and increased false positive rate by 11%[40]. The addition of other somatic mutations as p53 and p16 to K-ras mutation analysis has been shown to increase the sensitivity of PDAC detection to up to 100% in cases where FNA was inconclusive in one study[41]. Detection of chromosomal abnormalities by fluorescence in situ hybridization (FISH) analysis has also been recently investigated in the detection of pancreatic cancer. In combination with cytopathology, the use of FISH analysis to detect polysomy of chromosomes 3, 7, and 17 and deletion of 9p21 improves sensitivity of EUS-FNA from 61% to 85%[42]. Presently, in view of the high accuracy of standard FNA, together with elevated price and reduced availability of these genetic tests, it appears that its use in EUS-FNA samples should be limited to research protocols and in cases with inconclusive specimens.

EUS and staging of PDAC

Suspected malignant tumors of the pancreas should be assigned a TNM staging based on the most current American Joint Committee on Cancer staging classification, which describes the tumor extension (T), lymph node (N) and distant metastases (M) of tumors, respectively. If the tumor is limited to the pancreas, it is either a T1 or T2 lesion. If the tumor is smaller than 2 cm it is a T1, if it is larger is a T2. In case the lesion extends beyond the pancreas, it is either a T3 or T4 lesion. Tumors extending to the celiac artery or superior mesenteric artery are considered T4 lesions, and tumors involving any other of the surrounding pancreatic structures as portal vein, ampulla or duodenal wall but the celiac or superior mesenteric artery are classified as T3. The distinction between T3 and T4 is important, as T4 lesions with involvement of celiac or superior mesenteric arteries is considered unresectable for curative intent. Reported accuracies of T staging by EUS range from 63%-94%[4-6,8,11,43-57]. Nodal (N) metastases are classified as absent (N0) or present (N1); including peripancreatic, gastro-hepatic or celiac malignant appearing lymph nodes. The accuracy of EUS for N-staging of pancreatic tumors ranges from 41%-86%[4-6,8,11,44,58]. Malignant echofeatures for detection of metastatic lymph nodes include size greater than 1 cm, hypoechoic echogenicity, sharp distinct margins, and round shape. If a lymph node has all four echofeatures, there is an 80%-100% chance of malignant

invasion[59,60]. The sensitivity of EUS alone for the diagnosis of metastatic adenopathy in pancreatic PDAC is 28%-92%[5,6,44,49,51,52,54,55], however most report sensitivities under 65%. Metastatic lymph nodes that do not have all four endosonographic features described above[59] may therefore incorrectly assumed to be benign. Specificity of EUS alone for the diagnosis of metastatic adenopathy in PDAC is 26%-100%[5,6,44,49,51,52,54,55], however most report specificities above 70%. It is presumed that the addition of EUS-FNA of suspicious lymph nodes may increase specificity however there are little data that describe the impact of the addition of EUS-FNA to EUS alone. Routine EUS-FNA of peritumoral lymph nodes with pancreatic head cancers may not be necessary as those nodes are removed en-bloc with the surgical specimen. As presence of malignant celiac lymph nodes might preclude surgery, detailed survey of this region should be done at the time of preoperative EUS staging.

For detection of non-nodal metastatic cancer, CT and MRI are superior to EUS due to both anatomic limitations of normal gastrointestinal anatomy and the limited range of EUS imaging. Although the entire left and caudate hepatic lobes might be seen by EUS imaging in most patients, a portion of the right lobe may not be visualized by EUS. EUS clearly cannot replace but may supplement other modalities for staging of hepatic metastases. EUS might, however, detect and sample small hepatic lesions missed by other imaging modalities[61-63]. The sensitivity of EUS-FNA for benign and malignant liver masses reportedly ranges from 82%-94%[61,64] and the diagnosis of liver metastases from pancreatic cancer generally precludes surgical resection[64]. EUS may also identify and sample ascites either previously detected or undetected by other imaging studies[65,66]. Identification of malignant ascites and liver metastases by EUS-FNA is associated with poor survival following diagnosis[67]. Therefore, routine examination of the perigastric and duodenal spaces for ascites should be incorporated in the staging of every pancreatic mass.

THERAPEUTIC EUS APPLICATIONS

EUS and fiducials

Fiducials are inert radiographic markers implanted into a target tumoral lesion for both localization and tracking during image-guided radiation therapy (IGRT). This technique depends on reference points by which the lesion is identified and tracked during radiation therapy. Fiducials have been traditionally implanted by percutaneous or surgical approach. The use of EUS-guided fiducial placement was first described by Pishvaian *et al*[68] in a case series including 13 patients, 7 with PDAC. Technical success was achieved in 94%. Since then, several series reported successful EUS-guided implantation of fiducials in the pancreas (Figure 2), including more than 180 patients with technical success > 90%[38,69-73]. Reported complications were uncommon and included cholangitis in a case in which prophylactic antibiotics were not used),
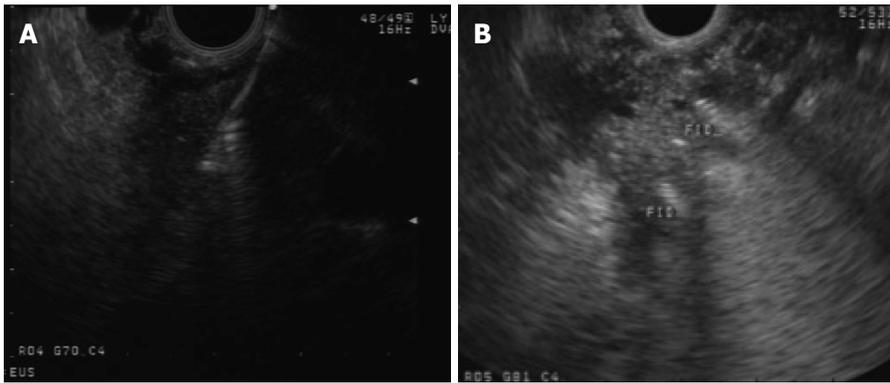
Figure 2 Endoscopic ultrasound guided deployment (A) and view (B) of fiducials in a pancreatic head mass.

mild pancreatitis, minor bleeding and fiducial migration requiring repeat procedure.

Traditional fiducials are cylindrical gold seeds that can be loaded in a 19G needle. More modern coil design fiducials can be loaded into a 22G needle and in theory coil design might reduce migration. However, this was not confirmed in a recent retrospective series including 39 patients with PDAC (103 fiducials). In this study, comparison of both types of fiducials showed no difference in migration and in addition traditional cylindrical fiducials were significantly more visible during IGRT[73]. In summary, EUS placement of fiducials appears a feasible and safe technique; its practice, however, will depend on local availability of EUS expertise and IGRT.

EUS and brachytherapy

Instead of placing an inert radiologic marker, brachytherapy involves the insertion of a radioactive seed directly into the pancreatic tumor for localized therapy. Currently the most common radioactive seed used clinically is iodine-125, which has a half-time of 59.7 d and tissue penetration of 1.7 cm[74]. Currently there are only 3 case series reporting EUS-guided brachytherapy in PDAC. In the pilot study by Sun *et al*[75], in 15 patients with advanced PDAC median survival was 10.6 mo, with 27% partial response and a mean number of 22 iodine-125 seeds per patient. Technical success was 100%; local complications including pancreatitis and pseudocyst occurred in 3 patients, also hematological toxicity without clinical sequelae occurred in 3 patients. In the subsequent series by Jin *et al*[76], a median number of 10 seeds were placed in 22 patients with advanced PDAC. Dose calculation was based on tumor volume from reconstructed three dimensional CT images. Although placement under EUS guidance was successful in all patients with no major complications, only three achieved partial remission at 4 wk and no improvement in survival was shown. However, pain was significantly reduced 1 and 4 wk after the procedure. The most recent larger series by Du *et al*[77] included 100 patients with advanced PDAC who underwent brachytherapy with EUS guided implanted iodine-125 seeds. Pain scores dropped dramatically after one week post implantation, and maintained significant lower until the

third month. The same group also used iodine-125 as a neurolytic agent in 23 patients undergoing EUS-guided CPN for unresectable PDAC[78]. At week 2.82% of patients had a reduction in pain score on a visual analogue scale and the mean narcotic consumption had decreased. This effect lasted until the study conclusion at 5-mo follow-up when only 2 patients were still alive. The authors postulate that iodine-125 may be a superior neurolytic agent compared to ethanol due to its longer half-life and deeper tissue penetration, although this has yet to be confirmed in a controlled clinical trial. The limited data so far for brachytherapy is encouraging, as it appears feasible and safe and might have some benefit in pain control in patients with locally advanced PDAC. Survival benefit, however, was not yet shown. Larger studies are needed to further evaluate this technique, including assessment of patient safety studies as well as safety of handling and storing radioactive material at endoscopy suites.

EUS-guided celiac plexus interventions

Patients with PDAC commonly develop abdominal pain that can be debilitating. Celiac plexus neurolysis (CPN) is a chemical splanchnicectomy of the celiac plexus that can be used to treat pain caused by PDAC. It can be performed by percutaneous, surgical or EUS-guided approach. EUS is well suited for identification of the celiac plexus due to the close approximation of the gastric wall with the origin of the celiac artery. EUS-CPN was first described in 1996 in 30 patients with intra-abdominal malignancy (25 with PDAC) who were treated with injection of bupivacaine and 98% absolute alcohol. Pain scores were significant lower compared with baseline at 2, 4, 8 and 12 wk after EUS-CPN[79]. Next, a prospective study including 58 patients with inoperable PDAC found that EUS-CPN provided significant decline in pain scores in 78% patients[80]. In a meta-analysis of randomized controlled trials of EUS-CPN for PDAC in 283 patients, Puli *et al*[81] reported 80% of patients experienced at least partial pain relief. Although the authors could not determine whether EUS-CPN reduced narcotic requirements due to heterogeneous reporting in the included studies, an earlier meta-analysis by Yan *et al*[82] reported a significant reduction in narcotic use with non-EUS guided CPN. Similar
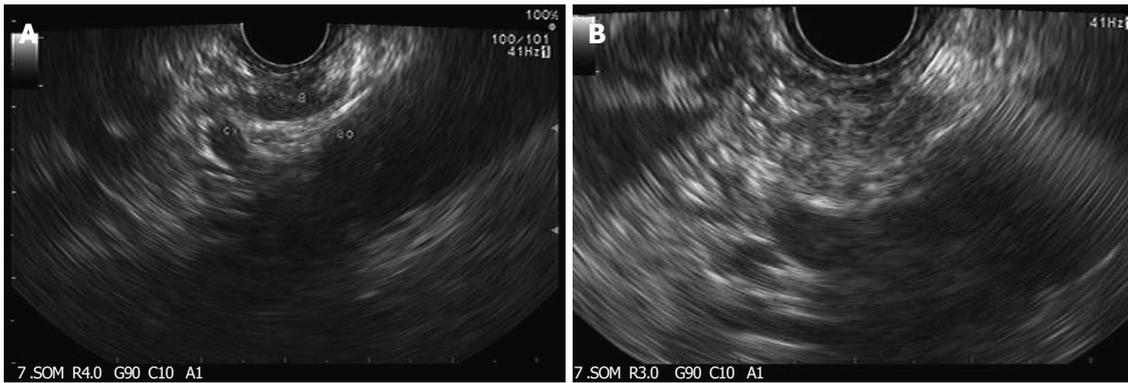
Figure 3 Celiac ganglia identified during endoscopic ultrasound (A) and injected during neurolysis under endoscopic ultrasound guidance (B).

findings were reported in a more recent Cochrane meta-analysis which combined studies evaluating EUS-guided and percutaneous CPN[83]. In a double-blind, controlled trial by Wyse *et al*[84], which included 96 patients with advanced PDAC, early EUS-CPN provided greater pain relief as compared with conventional therapy at 1 mo and significantly greater at 3 mo. Morphine consumption was similar in both groups at 1 mo but tended toward lower consumption at 3 mo in the neurolysis group.

Over the past decade, advancements in echoendoscope designs have permitted the accurate identification of celiac ganglia and interest has developed in direct ganglia injection to improve the efficacy of CPN (Figure 3A and B)[85]. In a recent randomized controlled study, celiac ganglion neurolysis was more effective than celiac plexus neurolysis in relieving pain (73.5% *vs* 45.5%, respectively; $P = 0.026$)[86].

EUS guided CPN is a safe procedure and complications are uncommon. Diarrhea (4%-15%) and orthostasis (1%) can occur due to disruption of the autonomic nervous system are usually mild and transient. A paradoxical increase in pain may occur in up to 9% of cases but generally resolves over several days[80]. Recently, serious complications including paralysis due to anterior spinal cord infarction[87,88], death from necrotic gastric perforation[89] or celiac artery thrombosis with infarction[90,91] have been reported.

EUS and fine needle injection
EUS-guided fine-needle injection (EUS-FNI) is an emerging method, which involves direct intra-tumoral delivery of therapeutic agents into pancreatic tumors under EUS guidance. This technique offers theoretic potential to deliver high dose concentration while minimizing systemic side effects.

In the pilot study by Chang *et al*[92], a single injection of allogeneic mixed lymphocyte culture (cytoimplant) was delivered by EUS-FNI in 8 patients with unresectable PDAC. The technique was feasible and not associated with substantial toxicity. In this series, median survival was 13.2 mo and two patients had partial response and one had a minor response. Subsequently, Hecht *et al*[93] reported the use of EUS-FNI of ONYX-015 (a

gene-deleted replication-selective adenovirus that preferentially targets malignant cells) in 21 patients with locally advanced PDAC without significant liver metastasis. Patients underwent 8 sessions of EUS-FNI and the final treatments were given in combination with systemic gemcitabine. In this study, mean survival was 7.5 mo, and there were 2 partial regressions, 2 minor responses and 6 patients with stable disease. Nevertheless, there were serious complications including 2 duodenal perforations and 2 patients with sepsis, therefore limiting the use of EUS-FNI of this agent.

EUS-FNI of immature dendritic cells was reported by Irisawa *et al*[94] in a series with 7 patients with metastatic PDAC who previously failed gemcitabine. Mean survival was 9.9 mo. There were 3 partial responses, 2 patients with stable disease and no serious reported complications. Also Hanna *et al*[95] reported EUS-FNI of BC -819, a DNA plasmid that has the potential to treat PDAC that overexpresses H19 gene, in 6 patients with advanced PDAC. There were 3 partial responses and no serious reported complications.

TNFerade biologic is a replication-deficient adenoviral vector that expresses tumor necrosis factor α (TNF-α) under control of the Egr-1 promoter, which is inducible by chemotherapy and radiation In a phase I / II study, EUS or percutaneously guided intra-tumoral TNFerade biologic with 5-fluorouracil and radiotherapy was well tolerated and showed promising results in 50 patients with locally advanced PDAC[93]. Successively, a randomized multicenter trial, TNFerade biologic was compared with standard of care (SOC) in 304 patients with locally advanced PDAC. TNFerade was injected intratumorally by either EUS-guided approach or percutaneous transabdominal approach. Results showed that the addition of TNFerade to SOC was well tolerated, however did not prolong survival in patients with locally advanced PDAC. In addition, in the TNFerade arm of the study, multivariate analysis showed that TNFerade injection by EUS approach, rather than a percutaneous transabdominal approach was a risk factor for inferior progression-free survival. It is possible that greater variability existing in EUS operator skill across the participating institutions compared to the more straight forward percutaneous

transabdominal approach technique might have resulted in reduced efficacy in the EUS group[96]. EUS-FNI although promising, up to now did not show noteworthy results in the treatment of PDAC.

EUS-guided biliary drainage

ERCP is the procedure of choice for bile duct stenting in obstructive jaundice in patients with advanced PDAC. When ERCP is not possible due to failed cannulation, altered upper gastrointestinal tract anatomy, a distorted ampulla, gastric outlet obstruction, a periampullary diverticulum or in situ enteral stents, EUS-guided biliary drainage (EGBD) has been used as a minimally invasive alternative to surgical biliary bypass or percutaneous transhepatic biliary drainage (PTBD)[97].

Two main approaches for EGBD have been used: direct transluminal stenting (hepaticogastrostomy or choledochoduodenoscopy, without accessing the papilla) and a rendezvous technique (wire placed into intrahepatic or extrahepatic biliary duct, passed through the papilla and retrieved by a duodenoscopy for biliary interventions). A third approach, EUS-guided antegrade transpapillary biliary stent placement, has also been described[98,99]. Case-series from expert tertiary centers suggest that EGBD can be performed with high therapeutic success (87%) but is associated with 10%-20% mild to moderate morbidity and rare serious adverse events[97,100-110]. Rendezvous technique appears to be the safest[110,111], however can only be attempted in whom the papilla or choledocho-enteric anastomosis is accessible by endoscopy. In addition, rendezvous biliary drainage either fail or is not possible in at least 25% of patients, is associated with prolonged procedure times and may lead to acute pancreatitis[97,102,107,108]. Transluminal stenting can be complicated by stent migration or occlusion, bile leak, cholangitis, hemobilia, pneumoperitoneum and bile peritonitis[104-106,111,112]. EUS-guided hepaticogastrostomy is potentially applicable to patients with duodenal obstruction or prior gastric surgery, however it can only be attempted when the left intra-hepatic system is dilated[111]. EUS-guided choledochoduodenostomy can be attempted only in patients with a native anatomy (intact duodenal bulb) and an intact biliary tree[112]. EGBD by using either rendezvous or directly transluminal technique requires needle puncture *via* an intrahepatic or an extrahepatic route in an non-obstructed patient with normal upper GI anatomy. It appears that extrahepatic route is preferable and safer than intrahepatic access, whether EGBD is performed by rendezvous or direct transluminal stenting[97,105,109,110].

Recently Park *et al*[110] reported a single-operator, non-randomized prospective study evaluating technical and functional success and adverse event rate of a treatment algorithm using a modified technique of "enhanced guidewire manipulation" for EGBD, performed at same-session after failed ERCP in 45 patients with malignant or benign biliary obstruction. Results of this approach showed a technical and functional success of 95% and overall adverse event rate of 11%, including pancreatitis, focal bile peritonitis, limited pneumoperitoneum, intra-

peritoneal stent migration and biloma.

Artifon *et al*[113] reported the first prospective randomized comparison between EGBD (choledochoduodenoscopy) and PTBD, in 25 patients with unresectable malignant biliary obstruction who failed ERCP (13 patients EGDB *vs* 12 patients in the PTBD group). In this small study, both groups had similar technical and clinical success, complication rate, cost and quality of life.

EGBD is a safe and effective alternative after failed ERCP, whether performed by rendezvous or direct luminal stenting. Although limited data suggests equivalency to PTBD, larger studies are needed to confirm those results. EGBD ideally should be performed by high skilled endoscopists trained in both ERCP and EUS, and should be limited to expert tertiary centers, where surgery and radiology back-up are available in case of adverse events.

CONCLUSION

EUS and EUS-FNA are highly accurate modalities for detection, diagnosis and staging of PDAC. This high accuracy is decreased, however in specific situations most notably in the presence of chronic pancreatitis. Newly techniques including contrast-enhanced EUS, elastography and detection of DNA markers are in progress and might improve the accuracy of EUS in the detection of PDAC in the setting of chronic pancreatitis. EUS and EUS FNA have recently progressed from a diagnostic to a therapeutic technique in the management of PDAC. Evolving therapeutic applications include celiac plexus interventions, fiducial and brachytherapy seeds placement, fine needle injection and EUS-guided biliary drainage. The future role of EUS and EUS in management of PDAC is still emerging.

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2014 ADVANCES IN PANCREATIC CANCER

Pancreatic biomarkers: Could they be the answer?

Angela Lamarca, Jaime Feliu

Angela Lamarca, Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester M20 4BX, United Kingdom
Angela Lamarca, Jaime Feliu, Department of Medical Oncology, La Paz University Hospital, 28046 Madrid, Spain
Author contributions: Lamarca A and Feliu J performed manuscript writing and reviewing.
Correspondence to: Angela Lamarca, MD, PhD, Department of Medical Oncology, The Christie NHS Foundation Trust, Wilmslow Road, Manchester M20 4BX,
United Kingdom. angela.lamarca@christie.nhs.uk
Telephone: +44-16-14468106 **Fax:** +44-16-14463468

Core tip: Implementing the clinicopathological information with molecular characteristics for treatment individualization in pancreatic cancer seems to be one of the keys to improving survival and response to treatment. The development of new biomarkers and a better definition of the current ones are radically important. This review will summarize the most important biomarkers defined for pancreatic adenocarcinoma and their current development status.

Abstract

Pancreatic ductal adenocarcinoma (PDA) is known for its poor prognosis. Most of the patients are diagnosed with advanced stages, when no curative treatment is available. Currently, despite extensive clinical research on PDA, the median overall survival remains short. Diagnosis delay and primary chemo-resistance due to its intrinsic biological nature may explain the challenges to improve our results. Our knowledge about the molecular biology of PDA has exponentially increased during the last decades and its use for the development of biomarkers could help to reach better results in the clinical setting. These biomarkers could be the clue for the improvement in PDA clinical research by earlier detection strategies with diagnostic biomarkers, and by an individualization of treatment approach with prognostic and predictive biomarkers. This review summarizes the current knowledge about the molecular biology of PDA and the status of the most important prognostic and predictive biomarkers.

Key words: Pancreatic adenocarcinoma; Biomarkers; Diagnosis; Prognostic; Predictive; Treatment

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is known for its aggressiveness and poor prognosis: it is the fourth leading cause of cancer-related death both in men and women[1]. Approximately 45220 patients are annually diagnosed with pancreatic adenocarcinoma; almost all are expected to die from the disease[2]. Five-year survival rate after the diagnosis is around 5% for all the stages, reaching 20% for the localized stages and being less than 1% for those patients diagnosed with advanced disease.

The majority of pancreatic tumours (85%) are classified as adenocarcinomas (PDA), arising from the ductal epithelium. The diagnosis is mainly made in patients in their forties and the incidence is higher in men than in women (ratio 1.3:1). Some risk factors have been suggested for the development of PDA, but no standard screening has been defined yet (Table 1). Five to ten percent of the patients diagnosed with PDA have a first degree relative with the same disease, which suggests involvement of familial aggregation and/or genetic factors[4].

Surgical resection is the only option of curative treatment. Nevertheless, because of the late presentation of

Table 1 Suggested risk factors for the development of pancreatic ductal adenocarcinoma[3]

Hereditary syndromes	Non-hereditary risk factors
Hereditary breast/ovarian cancer (BRCA2, BRCA1, PALB2)	Nonhereditary chronic pancreatitis
Familial atypical multiple mole melanoma (FAMMM) syndrome (CDKN2A)	Diabetes mellitus, glucose metabolism, and insulin resistance
Peutz-Jeghers syndrome (STK11)	Cigarette smoking
Familial adenomatous polyposis (APC)	Obesity and physical inactivity
Hereditary nonpolyposis colon cancer (Lynch Ⅱ) (DNA mismatch repair genes)	Diet (high intake of saturated fat and/or meat, particularly smoked or processed meats)
Familial pancreatic cancer (gene not identified)	Coffee and alcohol consumption
Hereditary pancreatitis (PRSS1, SPINK1)	Aspirin and nonsteroidal anti-inflammatory drug use
Ataxia telangiectasia (ATM)	History of partial gastrectomy or cholecystectomy
Li-Fraumeni syndrome (p53)	*Helicobacter pylori* infection

Table 2 Summary of the most important randomized clinical trials performed in advanced pancreatic ductal adenocarcinoma

Experimental arm treatment (number of patients included)	Median OS (mo) (Experimental arm) (95%CI)	Control arm treatment (number of patients included)	Median OS (mo) (Control arm) (95%CI)	Hazard ratio (95%CI) (*P* value)	Ref.
Gemcitabine (63 pts)	5.6 (data not shown)	5-FU (63 pts)	4.4 (data not shown)	Data not shown *P* = 0.0025	Burris *et al*[7], 1997
Gemcitabine and erlotinib (285 pts)	6.24 (data not shown)	Gemcitabine (284 pts)	5.91 (data not shown)	0.82 (0.69-0.99) *P* = 0.038	Moore *et al*[8], 2007
Gemcitabine and capecitabine (267 pts)	7.1 (6.2-7.8)	Gemcitabine (266 pts)	6.2 (5.5-7.2)	0.86 (0.72-1.02) *P* = 0.08	Cunningham *et al*[9] 2009
FOLFIRINOX (combination of 5FU, oxaliplatin and irinotecan) (171 pts)	11.1 (9.0-13.1)	Gemcitabine (171 pts)	6.8 (5.5-7.6)	0.57 (0.45-0.73) *P* < 0.001	Conroy *et al*[10], 2011
Gemcitabine and nab-paclitaxel (431 pts)	8.5 (7.9-9.5)	Gemcitabine (430 pts)	6.7 (6.0-7.2)	0.72 (0.62-0.83) *P* < 0.001	Von Hoff *et al*[11], 2013

OS: Overall survival.
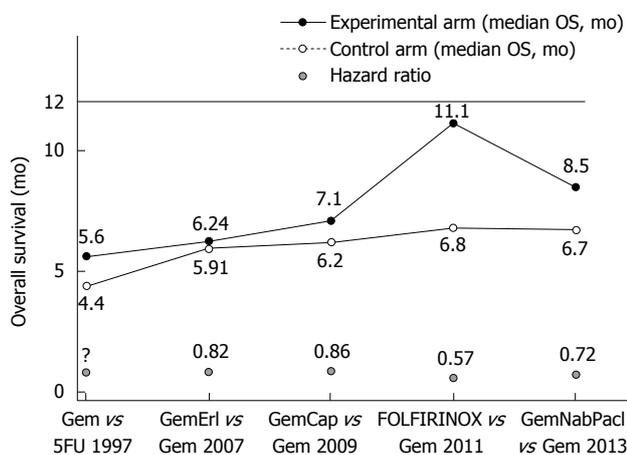
Figure 1 Multiple randomized phase III trials have been completed in the last decades; however, we have not been able to cross the barrier of 12 mo survival in advanced pancreatic cancer.

the disease, only 15%-20% of patients are diagnosed early enough to be considered for a potentially curative treatment. However, the relapse rate after surgery is high (80%-90%). Looking for a reduction in the relapse rate and an increase in the overall survival (OS), adjuvant chemotherapy is currently standard of care after resection of PDA. The most employed adjuvant chemotherapy schedules are gemcitabine or capecitabine[5,6].

Unfortunately, most of the patients (up to 80%) are

diagnosed in advanced stages and palliative chemotherapy is the only option of treatment. The aim of this chemotherapy is to prolong OS and improve the quality of life. In 1997, gemcitabine was established as the drug of choice for the treatment of advanced PDA with OS of 5.6 mo compared to 4.4 mo in the arm with 5FU[7]. Since then, multiple randomized studies with combination schedules have shown improvement in OS compared to single agent gemcitabine (Table 2)[8-11]. However, as it is shown in Figure 1, the impact in the survival achieved in advanced PDA has never reached the year of median OS. This is far of being comparable to the results achieved in other malignancies such as advanced breast or colorectal cancer.

WHY THESE RESULTS? WHAT ARE THE CHALLENGES WHEN TREATING PDA?

Much effort is been employed in trying to improve the survival of our patients with PDA. The improvement in the big randomized studies with more than 1800 patients during the last decades seems to be not enough and the median OS is still less than one year after diagnosis[12]. When we compare this data with other adenocarcinomas, for example breast or colorectal adenocarcinoma with median OS longer than 24 mo, we might wonder: are we doing the right research? What makes pancreatic cancer

Table 3 Core signalling pathways involved in pancreatic ductal adenocarcinoma

Involved pathways	PDA with pathway aberrations	Representative genes
Apoptosis	100%	*CASP10, VCP, CAD, HIP1*
DNA repair	83%	*ERCC4, ERCC6, EP300, RANBP2, TP53*
Regulation of G1/S phase	100%	*CDKN2A, FBXW7, CHD1, APC2*
Hedgehog pathway	100%	*TBX5, SOX3, LRP2, GLI1, GLI3, BOC, BMPR2, CREBBP*
Celular adhesion	79%	*CDH1, CDH10, CDH2, CDH7, FAT, PCDH15, PCDH17, PCDH18, PCDH9, PCDHB16, PCDHB2, PCDHGA1, PCDHGA11, PCDHGC4*
Integrin signaling	67%	*ITGA4, ITGA9, ITGA11, LAMA1, LAMA4, LAMA5, FN1, ILK*
c-Jun N-terminal kinase signaling	96%	*MAP4K3, TNF, ATF2, NFATC3*
KRAS signaling	100%	*KRAS, MAP2K4, RASGRP3*
Regulation of invasion	92%	*ADAM11, ADAM12, ADAM19, ADAM5220, ADAMTS15, DPP6, MEP1A, PCSK6, APG4A, PRSS23*
GTP-ase dependent signaling (not κ-ras)	79%	*AGHGEF7, ARHGEF9, CDC42BPA, DEPDC2, PLCB3, PLCB4, RP1, PLXNB1, PRKCG*
TGF-β pathway	100%	*TGFBR2, BMPR2, SMAD4, SMAD3*
Wnt/Notch pathway	100%	*MYC, PPP2R3A, WNT9A, MAP2, TSC2, GATA6, TCF4*

Addapted from Jones *et al*[19], 2008. PDA: Pancreatic ductal adenocarcinoma.

so hard to treat? Do we know enough about its molecular biology? Which is the next step?

Several reasons have been postulated for the difficulties in achieving better results in PDA[13]: (1) delay in diagnosis due to lack of symptoms until advanced stages. Most of the patients are diagnosed with distant metastases or unresectable locally advanced disease. Moreover, due to its location in the retroperitoneum, the pancreas is difficult to access and sample with traditional endoscopic techniques. This can also raise difficulties for an early diagnosis; (2) PDA is associated with several comorbidities that could affect patients' overall health with a worse impact in the OS of those who develop the PDA (Table 1); (3) limited effect of local therapies. The relapse rate is far from being acceptable, even with adjuvant chemotherapy or a combination of adjuvant chemo-radiotherapy. One possible explanation is that "field effect" mutations may affect normal appearing cells present in the residual pancreatic tissue. This, added to the high ability of spreading, even in early stages, could explain the high chances of relapse after local radical treatment[14]; and (4) PDA has been postulated to be primary (innate), rather than secondary (acquired), resistance to chemotherapy. Reasons for this could be both, related to the cancer cell itself and to the stroma surrounding the pancreatic cancer cells: (1) cancer cell characteristics. Different high penetrance genetic alterations have been described in PDA. One of the most frequent ones is activating mutations in κ-ras (present in > 90% of PDA), which is one of the most potent of all human oncogenes, and able to induce strong pro-growth, cell motility and invasion signals; and (2) a defining characteristic of PDA is the presence of a dense fibrotic proliferation surrounding the epithelial cells composed of various leukocytes, fibroblasts, endothelial cells and neuronal cells, as well as extracellular matrix components such as collagen and hyaluronan[15-17]. Moreover, in contrast to many tumours that are dependent on neo-angiogenesis, PDA is poorly vascularised and therefore, poorly perfused, making the delivery of chemotherapy more difficult into the tumour cells.

MOLECULAR BIOLOGY IN PANCREATIC CANCER: WHAT DO WE KNOW?

PDA is known to be a genetic disease, caused by inherited and acquired mutations in specific cancer-associated genes[18]. Since the sequencing of the protein-coding exons from 20661 genes in 24 advanced ductal adenocarcinomas of the pancreas was published in 2008, a better understanding of the key pathways involved in the development and maintenance of PDA was provided[19]. In 2012 the sequencing of 142 localized and resected PDAs was also published[20].

The most important genes and pathways involved in PDA biology are summarized in Table 3.

According to our current knowledge, multiple combinations of all these genetic mutations are commonly found in PDA, and can be classified as follows[21-24]: (1) mutational activation of oncogenes: predominantly K-ras; (2) inactivation of tumour suppressor genes such as *TP53, p16/CDKN2A*, and *SMAD4*; (3) inactivation of genome maintenance genes, such as *hMLH1* and *MSH2*, which control the repair of DNA damage. Most of these mutations are somatic aberrations. However, some germline aberrations were described (BRCA2, PALB2, STK11, ATM, MLH1 and MSH2) to be involved in the development of hereditary pancreatic cancer (Tables 1 and 3)[25].

During the last two decades, a lot of effort has been done in the definition of biological pathways involved, not only in the development/maintenance of PDA cancer cells, but also in the characterisation of the stroma surrounding the PDA cells[15-17]. As we discussed above, the characteristics of this particular stroma are one of the explanations for the difficulties in the treatment of PDA[26]. Some core pathways [*e.g.*, Hedgehog, Transforming growth factor (TGF)-β and Hepatocyte growth factor (HGF)-met] have shown to be involved in its development[18,27]. Moreover, some studies are testing the effec-

Table 4 Biomarkers in pancreatic ductal adenocarcinoma			
Biomarker	Prognostic biomarker	Predictive biomarker	Comments and references
MUC1	Yes		Predictive of early cancer-related death[37]
MSLN	Yes		Predictive of early cancer-related death[37]
6-gene signature	Yes		Expression of *FOSB, KLF6, NFKBIZ, ATP4A, GSG1* and *SIGLEC11* is related with metastatic spread[38]
VEGF	Yes		Worse survival in resected PDA[39]
p16	Yes		Higher expression was related to poorer prognosis[40]
TP53	Yes		Relation with tumour dedifferentiation and higher locorregional recurrence[40]
SMAD4	Yes		Higher Smad4/Dpc4 was related to bigger tumours, lymph node metastases and shorter survival[40]. Higher relapse rate (distant spread)[41]. Loss of expression correlated with resectability and better survival after surgery[42]
EGFR			No predictive/prognostic power[43,44]
K-ras	Yes		Better prognosis in Kras wild-type tumours[43,44]
RRM1	Yes	Yes	High expression of RRM1 showed significantly better overall survival[45-47] and worse response to treatment[47-49]
ERCC1	Yes		High ERCC1 expression showed significantly better overall survival[47,50,51]. No predictive power[49]
CTCs	Yes		More studies are awaited[52]
hENT1	Yes	Yes	High expression of hNENt1: worse prognosis, higher response to gemcitabine in the adjuvant setting; unclear impact in metastatic patients[50,53-57]
HuR	Yes	Yes	Low expression of HuR: worse prognosis[58] and better response to gemcitabine[59,60]
SPARC	Yes		Expression of SPARC in the peritumoural stroma is related with worse prognosis[61,62]. No predictive effect
CTGF			Preclinical data seem to suggest prognostic impact and potential predictive power for FB-3019[63-66]

VEGF: Vascular endothelial growth factor; EGFR: Epidermal growth factor receptor; CTCs: Circulating tumour cells; SPARC: Secreted protein acidic and rich in cysteine; CTGF: Connective tissue growth factor.

tiveness of anti-stroma therapies in pancreatic cancer, such as Visdemogib (Hedghog pathway inhibitor)[28,29] and nab-pacliaxel [postulated to be a secreted protein acidic and rich in cysteine (SPARC) inhibitor][30].

IMPROVING OUR RESULTS THROUGH THE DEVELOPMENT OF BIOMARKERS

A biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" by the Biomarkers Definitions Working Group[31]. According to this Working group, three categories of biomarkers can be defined depending on the information that they provide: diagnostic, prognostic and predictive biomarkers.

As detailed above, during the last decades, our knowledge about the PDA molecular biology is increasing exponentially and lots of pathways have been implicated in this malignancy. However, apart from CA19.9 for the diagnosis of pancreatic adenocarcinoma, no other biomarkers are currently being employed in PDA for improving the clinical management of these patients[24,32,33]. How can we apply all this new knowledge in the development of new studies looking for an improvement in OS? According to some experts, better early detection strategies with diagnostic biomarkers, treatment decisions based on prognostic biomarkers, and individualized treatment schedules based on predictive biomarkers could be the clue for the improvement in PDA research[34,35].

A lot of work has already been done in the development of a compendium of potential PDA biomarkers worth to be included in future research[36]. Nowadays, the three biomarker categories are being developed in PDA. The most important prognostic and predictors biomarkers for pancreatic cancer are summarized in Table 4.

Diagnostic biomarkers
The aim of the development of diagnostic biomarkers is to improve the rate of early diagnosis. CA19.9 is already employed as a diagnostic tool in combination with image techniques[23,30]. The definition of genetic expression and proteomic patterns could improve the diagnosis of PDA, currently based on morphological pathology studies only.

Prognostic biomarkers
The potential of classifying the patients into good and bad prognostic groups could be especially useful after surgery. We could offer more aggressive chemotherapy schedule or closer follow-up to those patients with worse prognosis or more chances of relapse. Moreover, the capability of defining the relapse pattern (local *vs* distant spread) could also improve the chosen image technique or frequency for the surveillance. See more details below.

Predictive biomarkers
The definition of predictive biomarkers, both for already employed drugs and for new therapies, could enrich our prospective studies. We need to improve our ability for selecting those patients that, according to the tumour expression of predictive biomarkers in PDA, may have better response to the chosen treatment and individual-

ize the chemotherapy according to this information. See more details below.

CURRENT DEVELOPMENT OF BIOMARKERS IN PANCREATIC CANCER

The most important prognostic and predictors biomarkers for pancreatic cancer are summarized in Table 4.

PROGNOSTIC BIOMARKERS IN PDA

To look for effective biomarkers able to stratify PDA based on biologic behaviour, a survival tissue microarray of 137 resected PDAs was analysed[37]. In a multivariate model, MUC1 (OR = 28.95, 3+ *vs* negative expression, *P* = 0.004) and MSLN (OR = 12.47, 3+ *vs* negative expression, *P* = 0.01) were highly predictive of early cancer-related death. In this study, MUC1 and MSLN were superior to pathologic features (tumour size, lymph node metastases, and nuclear grade) in predicting survival.

Stratford *et al*[38] identified a six-gene signature (*FOSB*, *KLF6*, *NFKBIZ*, *ATP4A*, *GSG1* and *SIGLEC11*) associated with metastatic disease. The results from the training set of 34 patients were validated in an independent series of 67 patients. The six-gene signature was independently predictive of survival and superior to established clinical prognostic factors such as grade, tumour size and nodal status (HR = 4.1, 95%CI: 1.7-10.0). Patients defined to be "high-risk" had a 1-year survival rate of 55% compared to 91% in the "low-risk" group.

In 2011 a meta-analysis of immunohistochemical markers in resected pancreatic cancer was published[39]. The aim of the study was to conduct a systematic review of the literature evaluating p53, p16, SMAD4, bcl-2, bax, vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) expression as prognostic factors in resected PDA. VEGF emerged as the most potentially informative prognostic marker (11 eligible studies, 767 patients, HR = 1.51, 95%CI: 1.18-1.92). Bcl-2, bax and p16 were also related to OS. Neither p53, SMAD4 or EGFR were found to have significant prognostic value.

The expression of hENT1, involved in the internalization of gemcitabine into the cancer cell, has been widely explored in PDA (See below, "predictive factors"). The prognostic value of the expression of hENT1 has been shown in several studies. Kim *et al*[32] reported in 2011 a series of eighty-four resected PDAs. Total RNA was isolated from paraffin-embedded tumours and the multivariate analysis confirmed the association of low expression of hENT-1 (*P* = 0.007) with worse OS and progression free survival (*P* = 0.016).

The genes *p16*, *TP53* and *SMAD4/DPC4* were included in a study by Jones *et al*[19] in 2008 as core pathways of PDA development and have been widely studied in PDA for its potential prognostic prediction.

A retrospective study published in 2013 aimed to clarify the implications of 3 major genes (*CDKN2A/p16*, *p53*, and *SMAD4/DPC4*) with clinico-pathological findings, including survival and patterns of disease progression, in 106 patients with resected PDA[40]. The expression of protein products of these genes was determined immunohistochemically. Genetic aberrations of these 3 genes were associated with malignant behaviour of PDA: a significant correlation was found between SMAD4/DPC4 immunolabeling and tumour size (*P* = 0.006), lymphatic invasion (*P* = 0.033) and lymph node metastasis (*P* = 0.006); loss of p16 immunolabeling (*P* = 0.029) and loss of SMAD4/DPC4 immunolabeling (*P* < 0.001) were significantly associated with shorter OS; and abnormal immunolabeling of p53 was significantly associated with tumour dedifferentiation (*P* = 0.022) and the presence of locoregional recurrence (*P* = 0.020).

Moreover, the expression of *SMAD4* has been analysed to define higher relapse rate and therefore worse prognosis, in several studies[40-42,67]. A study including patients with resected PDA analysed the expression of cell-cycle and cell-signaling involved key proteins using immunohistochemistry in a subgroup of 129 patients[42]. While aberrant expression of *p21*(WAF1/CIP1), cyclinD1, *p53* or *p16*(INK4A) was not associated with a difference in survival; loss of *SMAD4/DPC4* expression correlated with resectability (*P* < 0.0001) and was associated with improvement in survival after resection (*P* < 0.0001). In contrast, resection did not improve survival in patients whose tumour expressed *SMAD4/DPC4* (*P* = 0.5). The authors concluded that preoperative assessment of *SMAD4/DPC4* expression could be useful in the selection of patients that may benefit from surgical resection.

In 2011, Crane *et al*[41] reported the results of a phase Ⅱ clinical trial to assess the efficacy and safety of cetuximab, gemcitabine and oxaliplatin followed by cetuximab, capecitabine, and radiation therapy in locally advanced pancreatic cancer. Diagnostic cytology specimens were analysed for SMAD4/DPC4 protein expression (by immunohistochemistry). In this study, SMAD4/DPC4 protein expression correlated with local rather than distant disease progression (*P* = 0.016).

Finally, Iacobuzio-Donahue *et al*[67] performed rapid autopsies on 76 patients with documented pancreatic cancer. The histological features, the status of the *KRAS2*, *p53* and *SMAD4/DPC4* genes were correlated to the stage at initial diagnosis and patterns of failure (locally destructive *vs* metastatic disease). *SMAD4/DPC4* genetic status was highly correlated with the presence of widespread metastasis but not with locally destructive tumours (*P* = 0.007).

SPARC is expressed in the cell matrix and it is involved in cell matrix interactions, wound repair, cell migration and cancer growth regulation. The high expression of *SPARC* in the peritumoural stroma was defined as a worse prognostic factor both in localized and locally advanced patients[61,62]. The expression patterns of *SPARC* were characterized by immunohistochemistry in 299 resected pancreatic adenocarcinomas to evaluate the

Table 5 "Classic" predictive biomarkers for "classic" chemotherapies with potential interest in pancreatic cancer

Predictive biomarker	Drug	Theoretical impact[1]	Studies performed in pancreatic cancer (predictive outcome)?	Impact confirmed in pancreatic cancer?	Notes	Ref.
Thymidylate synthase	5FU	When negative, better response to 5FU	Yes	No	Predictive value in PDA not validated	[55,72-74]
DPD	5FU	When mutation DPD, more 5FU related toxicity	Yes	No	Survival benefit with S1 and DPD mutation	[73]
Topoisomerase I	Irinotecan	When positive, better response to Irinotecan	No	No	No data in pancreatic cancer	-
RRM1	Gemcitabine	When positive, better response to gemcitabine	Yes	Yes	Low expression correlates with better response	[47-49]
ERCC1	Oxaliplatin	When negative, better response to Oxaliplatin	Yes	No	No predictive effect	[49,51]
XRCC1	Oxaliplatin	When negative, better response to Oxaliplatin	No	No	No data in pancreatic cancer	-
EGFR/kras	Erlotinib	Erlotinib effective when EGFR mutation/kras wild type present	Yes	No	No predictive effect	[43,44]
PALB2	Mitomycin C	Mitomicin C effective when PALB2 mutation present	No	Yes	Case report	[75]
BRCA2	PARP inhibitors	PARP inhibitors effective when BRCA2 mutation present	Yes	Yes	Phase I trial	[76]

[1]This impact is suggested in other malignancies.

prognostic significance of tumoural and peritumoural *SPARC* expression[61]. In the multivariate analysis, *SPARC* expression in the surrounding stroma was a biomarker of worse prognosis (HR = 1.89, 95%CI: 1.31-2.74), while the expression of *SPARC* in pancreatic cancer cells remained unrelated to prognosis (HR = 1.02, 95%CI: 0.73-1.42). These data have been validated in further studies[62]. However, in animal models this prognostic impact of *SPARC* was not verified: the prognosis was worse in those *SPARC* knock-out mice[68].

Other prognostic biomarkers are: Kras[43,44] (better prognosis in Kras wild type tumours), HuR[59,60] (higher expression of HuR related to worse prognosis; See below "Predictive biomarkers"), RRM1[45-47] and ERCC1[47,50] (high RRM1 and high ERCC1 showed significantly better OS).

The analysis of circulating tumour cells is also being developed in PDA patients. However, further studies are awaited for a better understanding of its impact[52].

Collisson *et al*[69] reported in 2011 a study identifying three PDA molecular subtypes (classical, quasimesenchymal and exocrine-like) both in human tumours and cell lines, with different profiles of survival and response to treatment. The subtypes were defined according to the gene expression profile. These data need to be further validated but could be useful in the improvement of the individualize management of PDA[70].

PREDICTIVE BIOMARKERS IN PDA

Use of "classic" biomarkers to predict response to "classic" chemotherapies in pancreatic cancer

Based on data from other subtypes of adenocarcinoma such as colorectal tumour or breast cancer, some studies tried to employed "classic" predictive biomarkers to improve the results achieved with standard chemotherapy in advanced PDA. Although most of these biomarkers have

not been prospectively validated in pancreatic adenocarcinoma (Table 5), the rationale for this design is the individualization of first line chemotherapy according to the molecular expression profile of each tumour.

This idea has been executed in some studies. Von Hoff *et al*[71] published in 2012 the results of a clinical trial, where patients with PDA were treated according to molecular profiles of their tumour. The molecular analysis included immunohistochemistry, fluorescent in situ hybridization assays and immediately frozen tissue for oligonucleotide microarray gene expression assays. From the 86 patients included, there was a molecular target detected in 84 (98%) and 66 were treated according to the molecular profiling results. This was a pilot study, and the authors confirmed the feasibility of this rationale. However, prospective studies are ongoing and data are awaited for its wide use (NCT01726582, NCT01394120).

Gemcitabine response predictive biomarkers: RRM1, hENT1 and HuR

Apart from *RRM1*[47-49], whose lack of expression is predictive of response to gemcitabine (Tables 4 and 5), other biomarkers were suggested as predictors of response to gemcitabine in PDA: hENT1[50,53-55] and HuR[59,60].

Human equilibrative nucleoside transporter-1 (hENT1) was found to be the major gemcitabine transporter into the cell. Therefore, those cells with low expression of hENT1 will not transport the gemcitabine into the cancer cells, avoiding its activity (inhibition of the cell growth). In contrast, increased hENT1 abundance facilitates efficient cellular entry of gemcitabine and confers increased cytotoxicity. Nakano *et al*[77] reported in 2007 a preclinical study with pancreatic cancer cell lines where expression of hENT1 changed in the development of gemcitabine resistance.

However, interpreting these results in human samples is challenging. In patients receiving adjuvant treatment, the expression of hENT1 showed to be predictive biomarker for response to gemcitabine. However, this was not validated in the metastatic setting.

The multicentre ESPAC-3 trial randomized patients to adjuvant gemcitabine or 5FU after pancreatic adeno-carcinoma resection[78]. According to the safety profile, gemcitabine was chosen as the preferred agent when compared with monthly bolus (Mayo Clinic) 5-FU/LV for the adjuvant setting. The samples collected from the adjuvant ESPAC1/3 randomized trials were employed in a translational project to define the predictive value of hENT1[54]. One-hundred and seventy-five gemcitabine treated and 176 5-FU treated patients were included in the analysis. In the gemcitabine group a significantly lower survival ($P = 0.002$) was noted with low hENT1 [median survival 17.1 (95%CI: 14.3-23.8) mo *vs* 26.2 (95%CI: 21.2-31.4) mo]. Multivariate analysis confirmed hENT1 expression as a predictive biomarker of response to gemcitabine in the adjuvant setting.

However, the findings in metastatic patients are different. During the 2013 ASCO congress, data of a new gemcitabine-like drug (CO-101) were presented[57]. CO-101 (also known as CP-4126), a lipid-drug conjugate of gemcitabine, was rationally designed to enter cells independently of hENT1. The authors presented the results of a randomized trial comparing CO-101 and gemcitabine in the metastatic setting. The aim of the study was to determine whether CO-101 improved survival compared to gemcitabine in patients with low hENT1 tumours and to test prospectively the hypothesis that hENT1 was a predictive marker of response to gemcitabine. Unfortunately, CO-101 was not superior to gemcitabine in patients with low tumour hENT1 expression and, moreover, hENT1 expression did not predict gemcitabine treatment outcome in this study.

From these data, we conclude that while hENT1 seems to be a predictor of response in the adjuvant setting, this was not reproducible in metastatic patients. The molecular biology of the metastatic PDA may differ from the localized tumours, explaining the differences in the results.

The ubiquitous RNA-binding protein (RBP) HuR is involved in the control of gene expression, mRNA stability and translation and cellular response to internal and external signals[79]. Through its post-transcriptional effect by targeting mRNAs, HuR can alter the cellular response to proliferative, stress, apoptotic, differentiation, senescence, inflammatory and immune signals. The high expression of HuR has already been defined as a prognostic factor in PDA and some studies postulated HuR as a predictive biomarker for response to gemcitabine in cancer cell lines[59,60].

These results were confirmed in a series of 29 localized PDA patients in whom correlation between HuR expression levels and OS was evaluated[58]. The results indicated an increase in risk of death in patients with low

HuR levels compared to high HuR levels among patients receiving gemcitabine. Authors concluded that HuR was regulating the key metabolic enzyme for gemcitabine activation (deoxycytidine kinase) and could be a marker for therapeutic efficacy of gemcitabine based regimens: better response in patients with high HuR expression.

SPARC

As we detailed above, SPARC has prognostic impact in PDA[61,62]. Nab-paclitaxel is a 130-nm albumin-bound formulation of paclitaxel particles. Data from the phase I/II trial with nab-paclitaxel postulated SPARC as a predictive factor of anti-stromal therapies[30]. SPARC status was evaluated in 36 patients and a significant increase in OS was observed in high-SPARC expression subgroup compared with patients in the low-SPARC subgroup (median OS, 17.8 mo *vs* 8.1 mo, respectively; $P = 0.0431$). Moreover, some studies in animal models postulated that the addition of nab-paclitaxel could increase the intratumoural gemcitabine delivery due to anti-stromal effect of nab-paclitaxel[30,80]. However, the predictive impact of the expression of SPARC has not been clarified in the prospective studies with combination chemotherapy with gemcitabine and nab-paclitaxel[11,30].

Connective tissue growth factor/CCN2

Also focused in the stroma and the importance in PDA, connective tissue growth factor (CTGF) expression was analysed in pancreatic cancer. CTGF is a cysteine-rich matricellular secreted protein, which regulates diverse cell functions including adhesion, migration, proliferation, differentiation, survival, senescence and apoptosis[81,82].

Due to the hypoxic conditions surrounding the PDA, Eguchi *et al*[82] analyzed the tumour-stroma interaction signalling in cell lines of pancreatic cancer in hypoxia and normoxia using RNA interference techniques. The results showed that cell invasion was more enhanced under hypoxia than under normoxia ($P < 0.05$) and that CTGF was one of the overexpressed molecules in hypoxic conditions. Moreover, cell invasiveness was reduced by CTGF knockdown in hypoxic cancer cells ($P < 0.05$). The authors concluded that hypoxia induced CTGF expression could be a prognostic factor related to higher aggressiveness in PDA. This results match with those from other studies[63-65].

Therefore, the data available shows that CTGF is overexpressed in PDA and facilitates local desmoplasia, tumour survival and metastasis. FG-3019 is a human monoclonal antibody to CTGF, able to control the tumour growth in cancer cell lines[83] and tumour xenografts[65], without damage to the healthy tissue. Neesse *et al*[84] reported data from animal model research to clarify the antitumour effect of FB-3019. The authors concluded that FG-3019 may have antitumour effect itself, more than improving the delivery of gemcitabine into the tumour. First data in humans were presented in ASCO-GI 2013 by Picozzi *et al*[66], showing that the combination with gemcitabine, erlotinib and FG-3019 was safe in ad-

vanced pancreatic cancer patients. The authors showed that baseline CTGF plasma level was related to worse survival. Further clinical data for the prognostic and predictor relevance of CTGF in humans are awaited.

FUTURE, HOW TO IMPLEMENT THE ACTUAL DATA?

There is no doubt that the knowledge in molecular biology will continue to improve in the following years. New generation techniques are being employed in PDA research and will give much more data. However, it is crucial to incorporate this knowledge in a rational way, and this could be challenging. Moreover, the huge economic cost of this research needs to be analysed. Some panels of experts have defined the most suitable way for biomarker development and its addition to the clinical research in pancreatic cancer[12,85,86].

In conclusion, a lot of work needs to be done in the improvement of our understanding in pancreatic adenocarcinoma. Treatment individualization seems to be one of the keys, implementing the clinicopathological information with molecular characteristics. In order to achieve this, the development of new biomarkers and a better definition of the current ones are radically important. Most of the detailed biomarkers in this review are available just for research purposes; only Ca19.9 (with diagnostic and follow-up aim) is employed in the clinical practice. The results of the ongoing clinical trials with new biomarker research and the selection of the therapies according to these molecular characteristics are awaited.

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2014 ADVANCES IN PANCREATIC CANCER

Epigenetics and pancreatic cancer: Pathophysiology and novel treatment aspects

Daniel Neureiter, Tarkan Jäger, Matthias Ocker, Tobias Kiesslich

Daniel Neureiter, Institute of Pathology, Paracelsus Medical University/Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Tarkan Jäger, Department of Surgery, Paracelsus Medical University/Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Matthias Ocker, Experimental Medicine Oncology, Bayer Pharma AG, 13342 Berlin, Germany

Tobias Kiesslich, Department of Internal Medicine I, Paracelsus Medical University/Salzburger Landeskliniken (SALK), 5020 Salzburg, Austria

Tobias Kiesslich, Institute of Physiology and Pathophysiology, Paracelsus Medical University, 5020 Salzburg, Austria

Author contributions: All authors contributed equally in literature retrieval and evaluation, design and drafting of the manuscript.

Correspondence to: Daniel Neureiter, MD, MA, Institute of Pathology, Paracelsus Medical University/Salzburger Landeskliniken (SALK), Muellner Hauptstrasse 48, 5020 Salzburg, Austria. d.neureiter@salk.at

Telephone: +43-662-44824737 **Fax:** +43-662-4482882

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Abstract

An improvement in pancreatic cancer treatment represents an urgent medical goal. Late diagnosis and high intrinsic resistance to conventional chemotherapy has led to a dismal overall prognosis that has remained unchanged during the past decades. Increasing knowledge about the molecular pathogenesis of the disease has shown that genetic alterations, such as mutations of K-ras, and especially epigenetic dysregulation of tumor-associated genes, such as silencing of the tumor suppressor $p16^{ink4a}$, are hallmarks of pancreatic cancer. Here, we describe genes that are commonly affected by epigenetic dysregulation in pancreatic cancer *via* DNA methylation, histone acetylation or miRNA (microRNA) expression, and review the implications on pancreatic cancer biology such as epithelial-mesenchymal transition, morphological pattern formation, or cancer stem cell regulation during carcinogenesis from PanIN (pancreatic intraepithelial lesions) to invasive cancer and resistance development. Epigenetic drugs, such as DNA methyltransferases or histone deactylase inhibitors, have shown promising preclinical results in pancreatic cancer and are currently in early phases of clinical development. Combinations of epigenetic drugs with established cytotoxic drugs or targeted therapies are promising approaches to improve the poor response and survival rate of pancreatic cancer patients.

Core tip: Pancreatic cancer represents a devastating disease with poor overall survival at advanced stages, and new and effective treatment options are required. Besides genetic mutations, epigenetic dysregulation of oncogenes and tumor suppressor genes is recognized as a novel therapeutic target. Mechanisms underlying DNA methylation, histone acetylation and microRNA regulation and their contribution to pancreatic cancer development and resistance to treatment are highlighted in this review. Potential therapeutic interventions are discussed.

BRIEF INTRODUCTION TO PANCREATIC CANCER AND ITS CURRENT MODEL FOR PATHOGENESIS

Overall, the incidence of pancreatic cancer is minimally increasing or stable[1]. Experimental and clinical investigations have been intensified in the last few years (1) to obtain more pathogenetic insights in this highly life-destructive cancer entity; (2) to improve the early detection rate of this often concealed disease; and (3) to identify new therapeutic strategies to improve quality of life and survival time[2,3]. Nevertheless, the fate of patients with a diagnosis of pancreatic cancer is miserable even with maximal application of possible combined therapeutic interventions such as surgery, radiation and/or chemotherapy[4,5]. The overall survival time of patients with pancreatic cancer is a mean of 1 year after diagnosis[6]. This leads to the unsettling question of whether patients with a diagnosis of pancreatic cancer can survive at all[7].

In the last few years, one therapeutic point of attack has been concerned with the role of cancer stemcells and the epithelial-mesenchymal transition under the influence of epigenetic regulator mechanisms[8]. These approaches are interesting and promising as they could explain the chemotherapy refractiveness of most pancreatic cancers. We have shown previously that pancreatic cancer employs classical pathways of organ development and embryogenesis such as Hedgehog or WNT (wingless) signaling[9,10] which, amongst others, could be targets for novel therapeutic approaches.

In this review, we focus on epigenetic regulation mechanisms in pancreatic cancer providing possible novel treatment aspects by highlighting the pathophysiology of this special tumor entity for pathologists, clinicians and future therapeutic approaches.

Epidemiologic (re-)view of pancreatic cancer

Pancreatic cancer is associated with a high mortality rate and represents the 7th most frequent cause of cancer death, with approximately 265000 deaths and an incidence of 280000 per year worldwide in 2008[11,12]. Europe and Northern America have the highest incidence of pancreatic cancer, with slightly more males being affected.

Pancreatic cancer is usually diagnosed at an advanced stage due to a lack of symptoms in the early stages so that resection of the advanced tumor is often not possible. The overall 1-year survival rate for pancreatic cancer is 26%, and the 5-year survival rate is approximately 6% for advanced cancer and 22% for early stages when surgical removal of the tumor is still possible[13].

Therefore, new therapeutic approaches combining neoadjuvant chemotherapy and radiotherapy to significantly reduce the tumor size are promising to allow the option of surgical removal in selected patients[14].

Morphological aspects under respect of the precursor lesions

Classical malignant pancreatic tumors show heteroge-neous glandular and duct-like, grading-dependent structures, mostly infiltrating the pancreatic parenchyma and exhibiting a partially prominent desmoplastic stroma. The widely accepted and routinely used grading system of pancreatic cancer is based on (1) glandular differentiation; (2) mucin production; (3) mitosis (per 10 microscopic high power fields), and (4) nuclear features (such as nuclear polymorphism, size or arrangement of the nucleus)[15,16]. So far, no definitive and routinely used immunohistochemical markers exist, although many biological markers in pancreatic ductal adenocarcinoma were tested as possible diagnostic and prognostic tools. However, the main limitations arise from the small number of patients studied and in the heterogeneity of the applied methods[17]. Further approaches for prognostic grading focused on different morphological patterns similar to Gleason's scoring system[18], included epithelial-mesenchymal characteristics, such as vimentin expression and tumor budding[19,20], or evaluated several gene expression signatures including downregulation of ASPM (abnormal spindle-like microcephaly associated) which could be detected by immunohistochemistry[21].

Detailed morphological analysis revealed prognostic subtypes of pancreatic cancer, with a group associated with better survival (colloid and medullary) and a group with a worse outcome (adenosquamous or undifferentiated)[22] (as described in Table 1).

It is important not only to give the diagnosis "pancreatic cancer", but to discriminate between tumor entities for patient communication, and to evaluate a possible family history of cancer in genetic counselling (as in cases of medullary carcinoma[23]) as well as to establish tumor-specific therapy modalities, since it is possible to link tumor sub-entities to specific genetic lesions (Table 1).

Pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) are considered precursor lesions[24] (Figure 1). As IPMN and MCN are seen pre-therapeutically in radiological investigations, therapeutic strategies/algorithms were established to weigh up the extent of surgical resection and patients' quality of life.

Further molecular analyses of different precursor lesions and their morphological variants revealed a stepwise model of carcinogenesis and possible epigenetic associations (Figure 1). Interestingly, a huge number of epigenetically regulated genes have been detected by global gene expression profiles in comparison with classical genetic alterations showing an association with cytological and architectural atypia of these precursor lesions. The challenge in the future is to analyze the complex mechanistic crosstalk of these genetic and epigenetic regulatory mechanisms[25] during pancreatic carcinogenesis for new drug development and administration, as shown by Breitkreutz *et al*[26] using network statistics.

Molecular aspects of pancreatic cancer: Oncogenes and suppressor genes

Intensive DNA analysis using genome- and epigenome-wide screening methods during the last few years have

Table 1 Histomorphologic subtypes of pancreatic cancer related to their genetic/epigenetics and possible prognostic characteristics			
Variant (source)	**Morphology**	**Genetics/epigenetics**	**Prognosis**
MC	Well defined pushing border, syncytial growth pattern, and poorly differentiated cancer cells	Germline or somatic mutations as well as epigenetic silencing by promoter methylation of mismatch repair genes *MLH1* and *MSH2*	Better MC: Overall 2- and 5-yr survival rate of 29% and 13%[23]
CC	Suspension of well-differentiated cancer cells in extracellular mucin pools (at least 80%)	Unknown	CC: 2-yr and 5-yr survival rate of 70% and 57%[153]
AC	Combination of glandular and squamous (at least 30%) components	*K-RAS2* mutations, inactivation of *CDKN2A/p16*, *SMAD4/DPC4* and *TP53*	Worse AC/UC: Median survival of 5 mo after resection[154,155]
UC	Noncohesive cancer, lacking histological features of differentiation	*K-RAS2* gene mutations, loss of E-cadherin protein expression (promoter hypermethylation) Expression of *L1CAM*, *COX2*, and *EGFR* Subtype with osteoclast-like giant cells shows mutations like noninvasive precursor lesions	

MC: Medullary carcinoma; CC: Colloid carcinoma; AC: Adenosquamous carcinoma; UC: Undifferentiated carcinoma; COX: Cyclooxygenase; L1CAM: L1 cell adhesion molecule; EGFR: Epidermal growth factor receptor; MSH2: MutS homolog 2.

	PanIN	IPMN	MCN
Typical histology			
Major morphological hallmark(s)	Microscopic papillary or flat non-invasive epithelial neoplasm with different cytologic and architectural atypia	Mucin-producing epithelial neoplasm with predominant papillary architecture (arising from the main pancreatic duct or branch duct)	No connection to the pancreatic duct-associated ovarian-type stroma (mainly women)
Major genetics depending on grade of dysplasia	PanIN 1: Telomere shortening ↑, K-RAS2 ↑ PanIN 2: CDKN2A/p16 ↓ PanIN 3: TP53 ↓, SMAD4/DPC4 ↓, BRCA2 ↓	Low grade: K-RAS2 ↑ High grade: CDKN2A/p16 ↓, TP53 ↓	Overall not well defined Non invasive: K-RAS2 ↑, p53 ↑ Invasive: SMAD4/DPC4 ↓
Frequently hypermethylated genes (details see[156-161])	NPTX2, LHX1, RPRM	RELN, TFP2, CADM1/TSLC1, UCHL1	
	CLDN5: IPMN > PanIN, PENK: IPMN > PanIN		
	SPARC/ON: IPMN > PanIN, SFRP1/SARP2: IPMN > PanIN		
	CDKN2A/p16: IPMN, MCN > PanIN		

Figure 1 Precursor lesions of pancreatic cancer with their typical morphologic and genetic as well epigenetic characteristics. PanIN: Pancreatic intraepithelial lesions; IPMN: Intraductal papillary mucinous neoplasms; MCN: Mucinous cystic neoplasms; RELN: Reelin.

elucidated some major deregulated gate "drivers", "passengers" and "keepers" in pancreatic cancer (Table 2)[27-30]. Overall, more suppressor genes than oncogenes are involved in pancreatic cancer.

As recently described in depth by Hong *et al*[31], the most frequently mutated oncogene (> 95%) in pancreatic cancer is K-RAS (Kirsten rat sarcoma viral oncogene homolog), leading to constitutive downstream signaling of proliferation, cellular survival, motility and remodeling. On the other hand, the major deregulated suppressor genes in pancreatic cancer are *CDKN2A/p16* (cyclin-dependent kinase inhibitor 2A), *TP53* (tumor protein p53) and *SMAD4/DPC4* (SMAD family member 4) which are inactivated by 2 different, often independent, mechanisms. Whereas *CDKN2A/p16* and *TP53* are

mainly involved in cell cycle checkpoint control and arrest, *SMAD4/DPC4* plays an important role in signal transduction of the transforming growth factor (TGF)-β pathway, and furthermore in cellular proliferation. Finally, when looking at epigenetically affected genes, it is obvious that the classical and most frequent pancreatic cancer genes are only partially epigenetically regulated (see Table 2). Interestingly, such cases with epigenetically silenced *MLH1* (mutL homolog 1) genes are associated with the distinctive medullary phenotype of pancreatic cancer[32-34].

Linking morphology and genetics to epigenetics in pancreatic cancer

As later described in detail (see Figure 1, Tables 3 and 4), epigenetics *via* DNA methylation, histone acetylation or

Table 2 Activated and deactivated key genes in pancreatic cancer according to hallmark of cancer, frequency as well as to kind of possible genetic and epigenetic alteration

Gene symbol (source)	Associated function according the hallmarks of cancer[1][35,36]	Frequency	Type of genetic alteration	Evidence for epigenetic regulation (reference)
Activation				
K-RAS2[131]	a, f, g	> 90	Point mutation	
AKT2[162,163]	a, j, g	10-20	Amplification	
BRAF[164]	a, b, g	5	Point mutation	
Deactivation				
CDKN2A/p16[165]	b, d, i	95	Homozygous deletion, intragenic mutation	Yes[166]
TP53[167-169]	b, d, i, h	50-70	Intragenic mutation an one allele and loss in the other allele	
SMAD4/DPC4[170,171]	b, c, f	55	Homozygous deletion, intragenic mutation	
MLH1[23,172]	h	3-15	Heterozygote mutations	Yes[34]
BRCA2[173]	a	7	Heterozygote mutations	
STK11/LKB1[174]	i	5	Homozygous deletion, intragenic mutation	
TGFBR2[175]	a, f	4	Homozygous deletion, homozygous frameshift mutation	
MAP2K4[176,177]	a	2	Homozygous deletion, missense mutation	

[1]Hallmarks of cancer: Sustaining proliferative signaling (a), evading growth suppressors (b), avoiding immune destruction (c), enabling replicative immortality (d), tumour promoting inflammation (e), activating invasion and metastasis (f), inducing angiogenesis (g), genome instability and mutations (h), resisting cell death (i), deregulating cellular energetics (j)[35,36].

Table 3 Overview of DNA hyper-/hypomethylation involved in pancreatic cancer

DNA modification	Material			Gene affected	Ref.
	Cancer samples	Cell lines	Other		
DNA hyper-methylation	√			*p16*	[166]
	√			*RASSF1A*	[178]
	√			*MDFI, hsa-miR-9-1, ZNF415, CNTNAP2, ELOVL4*	[179]
	√			*SOX15*	[180]
	√			HOP hoemobox (*HOPX*)	[181]
	√			*KLF10*	[182]
	√			*hMLH1*	[183]
	√			*miR-34a/b/c*	[184]
	√			*SPARC*	[185]
	√			*FoxE1, NPTX2, CLDN5, P16, TFPI-2, SPARC, ppENK*	[186]
	√			*SFRP*	[187]
	√	AsPC1, Hs766T, MiaPaCa2, Panc1		*UCHL1, NPTX2, SARP2, CLDN5, reprimo, LHX1, WNT7A, FOXE1, TJP2, CDH3, ST14*	[30,159]
	√	AsPC1, BxPC3, CFPAC1, Panc1		*NPTX2*	[188]
	√	Panc1, SW1990		*miR-132*	[121]
	√	BxPC3, Capan2, CFPAC1, HPACl, HPAF Ⅱ, MiaPaCa2, Panc1, PL45		*FOXA1/2*	[81]
		MiaPaCa2		*ARID1B*	[189][1]
		Panc1		*NPTX2*	[190][1]
		AsPC1, BxPC3, Panc1, MIA PaCa-2		*Dkk3*	[191]
		BXPC3, HPAF Ⅱ, HPAC, hTERT-HPDE, Panc1		*Cldn18*	[192]
		BxPC3, CFPAC1, Panc1, SW1990		*TNFRSF10C*	[193]
			Pancreatic juice	Neuronal pentraxin Ⅱ (*NPTX2*)	[194]
			Pancreatobiliary fluid	*UCHL1, RUNX3*	[195]
			PanIN	*p16*	[196]
			IPMNs	*BNIP3, PTCHD2, SOX17, NXPH1, EBF3, SPARC, SARP2, TSLC1, RELN, TFPI2, CLDN5, UCHL1*	[157,197]
			Blood, brush cytology	*NPTX2*	[198,199]
DNA hypo-methylation	√			*VAV1*	[79]
	√			Claudin4, lipocalin2, 14-3-3 sigma, trefoil factor 2, S100A4, mesothelin, prostate stem cell antigen	[78]
	√			*MUC4*	[77]
		SW1990		*ABCB1/MDR1, ABCC1/MRP1, ABCG2/BCRP*	[200]

[1]Only abstract available.

Table 4 Overview of miRNAs associated with specific targets/functions in pancreatic cancer

miRNA/function		Cell lines	Target gene(s)	Cellular effects	Ref.
Function as oncogene	-10a	AsPC1, Capan1, Capan2, MiaPaCa2, Panc1, Patu8988T, Patu8988S, Patu8902	*HOXB1, 3*	Metastasis ↑	[201]
	-21	AsPC1, BxPC3, Capan1, Capan2, CFPAC1, Hs776T, H48N, KP-1N, KP-2, KP-3, MiaPaCa2, NOR-P1, Panc1, SUIT-2, SW1990	*HOXA1*	Invasion ↑	[202]
		AsPC1, Capan1, Capan2, CFPAC1, H48N, HS766T, KP-1N, KP-2, KP-3, MiaPaCa2, NOR-P1, Panc1, SUIT-2, SW1990		Proliferation ↑, invasion ↑, chemoresistance ↑	[203]
		BxPC3		Proliferation ↑	[204]
		Capan1, HS766T, MiaPaCa2, MPanc96, Panc1, PL45, SW1990	*PTEN, RECK*	After miRNA inhibition: cell cycle arrest ↑, apoptosis ↑	[205]
	-132, -212	Panc1	*Rb1*	Proliferation ↑	[206]
	-155	Capan2, MiaPaCa2, MCF7, MEFs, 293T	*TP53INP1*	Apoptosis ↓	[207]
	-194, -200b, -200c, -429	AsPC1, A818, BxPC3, Capan1, Capan2, HPAFII, MiaPaCa2, MPanc96, Panc1, Patu8902, Patu8988T, Patu8988S, PT45, Suit 007, Su.86.86, Sut0028[1]	*EP300*	Metastasis ↑	[208]
	-197	AsPC1, Panc1	*p120 catenin*	EMT ↑	[209]
	-210	Panc1, MiaPaCa2, SUIT-2		Migration ↓, vimentin ↓, snai-1 ↓, membraneous β-catenin ↑	[210]
	-221	Capan1, HS766T, MiaPaCa2, MPanc96, Panc1, PL45, SW1990	*p27*	Chemosensitivity ↑	[205]
	-224, -486	AsPC1, A818, BxPC3, Capan1, Capan2, HPAFII, Su 86.86, MPanc96, MiaPaCa2, Panc1, Patu8902, Patu8988T, PT45, Patu8988S, Suit 007, Suit 0028[1]	*CD40*	Invasion ↑, metastasis ↑	[211]
Function as tumor suppressor	-301a	BxPC3, Hs766T	*Bim*	Proliferation ↑,	[212]
	-320c	AsPC1, Panc1[2]	*SMARCC1*	Chemoresistance ↑	[213]
	-421	SW1990, Panc1	*DPC4/Smad4*	Proliferation ↑, colony formation ↑	[214]
	-491-5p	AsPC1, Capan1, MiaPaCa2, SW1990	*Bcl-XL, TP53*	Proliferation ↓, apoptosis ↑, STAT3 ↓, PI-3K/Akt ↓	[215]
	let-7	BxPC3, Capan1, Capan2, human HPNE (human pancreatic nestin-positive) cells MiaPaCa2, Panc1		Proliferation ↓, K-RAS ↓, MAPK ↓	[216]
	let-7a	AsPC1	*RAS*	K-RAS ↓, radiosensitivity ↑	[217]
	-22	BxPC3	*SP1, ESR1*	Tumourigenesis ↓	[218]
	-26a	SW1990, Panc1	*HMGA1*	Proliferation ↓, invasion ↓, migration ↓, apoptosis ↑	[219]
	-34	BxPC3, MiaPaCa2	*Bcl-2, Notch-1/2*	Clonogenicity ↓, invasion ↓, apoptosis ↑, cell cycle arrest ↑, chemosensitivity ↑, radiosensitivity ↑, CSC ↓	[220]
	-34a	Panc1		Cell cycle arrest ↑, apoptosis ↑, migration ↓, E2F3 ↓, Bcl-2 ↓, c-myc ↓, cyclin D1 ↓	[221]
	-34b	AsPC1, MiaPaCa2	*Notch-1 Smad3*	Proliferation, apoptosis Progression *in vivo* ↑	[222] [223][3]
	-107	MiaPaCa2, Panc1	*CDK6*	Proliferation ↓	[224]
	-126	AsPC1, BxPC3, KLM-1, MiaPaCa2, Panc1	*ADAM9*	Migration ↓, invasion ↓, E-cadherin ↑	[225]
	-132	BxPC3, HPAFII, HPAC, Panc1		Proliferation ↓, colony formation ↓, Akt ↓	[121]
	-143	AsPC1, BxPC3, Capan2, HPAFII, MiaPaCa2, Panc1	*COX-2*	Proliferation ↓, MEK/MAPK ↓	[226]
		Panc1	*ARHGEF1 (GEF1), ARHGEF2 (GEF2), K-RAS*	Migration ↓, invasion ↓, metastasis ↓, E-cadherin ↑	[227]
	-148a	IMIM-PC2	*CDC25B*	Proliferation ↓, colony formation ↓	[228]
	-148b	AsPC1, BxPC3, MiaPaCa2, Panc1, SW1990	*AMPKα1*	Proliferation ↓, apoptosis ↑, cell cycle arrest ↑, invasion ↓, chemosensitivity ↑, tumourigenicity ↓	[229]
	-150	Colo357, HPAF, Panc10.05	*MUC4*	Proliferation ↓, clonogenicity ↓, migration ↓, invasion ↓, cellular adhesion ↑	[230]

-200	AsPC1, BxPC3, Colo357, HPAC, MiaPaCa2, L3.6pl, Panc1[2]	EMT ↓ (ZEB1 ↓, slug ↓, vimentin ↓)	[231]
-375		Proliferation ↓, cell cycle arrest ↑, apoptosis ↑	[232][3]
-548d	Panc1	Proliferation ↓, apoptosis ↑, cell cycle arrest ↑	[233]

[1]Including an orthotopic murine model; [2]Gemcitabine-resistant cell line; [3]Only abstract available. Based on and updated from Park *et al*[120] 2011.

Table 5 Overview of Epigenetic mechanisms - see text for details and references

Mechanism	Enzyme	Subclasses/components	Effect on target gene expression
DNA (de-) methylation	DNMT	DNMT1 (methylation maintenance)	↓
		DNMT3A and -3B (*de novo* methylation)	
	DNA de-methylase	Not known	↑
Histone (de-) acetylation	HAT	*e.g.*, CBP, p300	↑
Histone methylation	HDAC	Class Ⅰ (HDACs-1-3, -8), class Ⅱa (HDACs-4, -5, -7, -9), class Ⅱb (HDACs-6, -10), class Ⅲ (SIRT1-7), class Ⅳ (HDAC-11)	↑
	PcG→ H3-K27-me3	PRC1: CBX-2, 4, or 9, PHC-1, 2, or 3, BMI1, RING1A/B or RNF2 → H3-K27-me3 maintenance	↓
		PRC2: EZH2, SUZ12, EED → *de novo* H3-K27-me3 maintenance	
	TrxG → H3-K4-me3	Several members	↑
Post-transcriptional	miRNAs	2578 mature miRNA (miRBase v20)	↓

HAT: Histone acetylase; HDAC: Histone deactylase; DNMT: DNA methyltransferase.

interacting regulative microRNAs (miRNAs) could essentially be linked to different morphological and genetic changes during pancreatic carcinogenesis. Extensive investigations are been carried out on epigenetic changes in pancreatic cancer precursor lesions, indicating that heterogeneous, non-linked pathways of carcinogenesis are regulated by epigenetics as summarized in Figure 1 and Table 3. Detailed analysis of the function of these epigenetically deregulated genes revealed that all hallmarks of cancer[35,36] such as self-sufficiency in growth signals (*e.g.*, SFRP1/SARP2; secreted frizzled-related protein 1), insensitivity to anti-growth signals [*e.g.*, CDKN2A/p16 or RPRM (reprimo)], tissue invasion and metastasis [*e.g.*, SPARC/ON (secreted protein, acidic, cysteine-rich (osteonectin)], limitless replicative potential [*e.g.*, LHX1 (LIM homeobox 1)], sustained angiogenesis [*e.g.*, CLDN5 (Claudin-5)] or evading apoptosis [*e.g.*, RPRM or CADM1/TSLC1 (cell adhesion molecule 1/tumor suppressor in lung cancer 1)] are involved in pancreatic cancer and affected by epigenetic (de)regulation. This supports our knowledge of the pleiotropic effects of systemic epigenetic mechanisms. The degree of cytological and architectural atypia correlated with the amount of methylated genes supporting the hypothesized multiple step model of pancreatic cancer even in the early disease stages[31,37].

Additionally, hypomethylation is also recognized in pancreatic cancer, leading to genomic instability by overexpression of genes and proteins in contrast to hypermethylation by silencing genes and subsequent protein expression. Serial genes like *SERPINB5* (serpin peptidase inhibitor, clade B, member 5), *CLDN4*, stratifin, lipocalin-2, trefoil factor2, *S100P* (S100 calcium-binding protein P), mesothelin or prostate stem cell antigen are hypomethylated which causes uncontrolled or "dys"-regulated cell cycle progression, proliferation, differentiation or adhesion[31].

EPIGENETICS OF PANCREATIC CANCER
Overview of epigenetic mechanisms
The identification of DNA methylation, histone modification and the action of miRNAs has profoundly increased the knowledge about the regulation of gene activity. Epigenetics studies the stable and inheritable patterns of altered gene expression independent of the primary DNA sequence[38], and has shown that dynamic traits of chromatin, reversible covalent modification of DNA, and post-transcriptional regulation centrally impact on gene expression and phenotypic characteristics[8,39]. With increasing evidence that tumorigenesis-associated cellular changes are caused by epigenetic alterations, the field of cancer research has evolved to incorporate oncogenic mechanisms beyond DNA mutations. Epigenetic mechanisms (see Table 5 for an overview about the major epigenetic mechanisms) are generally reversible. Together with the fact that epigenetic alterations may be even more prevalent than genetic aberrations, this is highly attractive in the conceptual approach of selecting and exploiting potential molecular targets for novel cancer therapeutics[8,40].

The methylation of DNA and subsequent silencing of a gene is catalyzed by DNA methyltransferases (DNMTs) which add a methyl group to the 5' carbon of the cytosine pyrimidine ring. This occurs preferably in regions containing cytosine-guanine dinucleotides (CpGs); these CpG islands are preferentially located in regions corresponding to regulatory regions of many genes[41].

While DNMT1 is responsible for maintenance of parental DNA methylation patterns following replication, *de novo* DNA methylation is catalyzed by DNMT3A and DNMT3B enzymes[42]. The identification of DNA demethylases which remove the methyl group and reverse the action of DNMTs still warrants further research. DNA methylation was the first type of epigenetic alteration identified as responsible for inactivation of a tumor suppressor gene[43], and it is suggested that 100-400 hypermethylated CpG islands may exist in a given tumor[44].

Compared with DNA-based epigenetics, alterations in DNA-associated histones offer a greater variety of covalent epigenetic modifications, including phosphorylation, methylation, acetylation, ubiquitination and sumoylation, all with different degrees of modification (*e.g.*, mono-, di-, and trimethylation)[45,46]. The nucleosome as the core building block of eukaryotic chromatin comprises histone octamers (dimers of H2A, H2B, H3 and H4, *i.e.*, the nucleosome core particle, NCP) and about 146 base pairs of DNA[47]. Modifications to histones determine the packing of DNA into either tight and transcriptionally silent heterochromatin or transcriptionally active and open-structured euchromatin. These modifications affecting chromatin mobility and stability have been termed "marks" whose type, position, and combinations determine whether a given gene is expressed or silenced, *i.e.*, the histone code[46,48]. This hypothesis postulates that local histone modifications determine the configuration of chromatin, alter the binding affinities of non-histone proteins, and subsequently influence the chromatin structure and accessibility of DNA for transcription[39,49]. Histone acetylation is thought to activate gene transcription and is catalyzed by histone acetyltransferases (HATs) which transfer an acetyl group from acetyl coenzyme A to the ε-amino group of lysine such as CREBBP (cAMP response element-binding protein binding protein), p300 and p300-CBP-associated factor (P/CAF). The reverse reaction is mediated by histone deacetylases (HDACs) which comprise a group of 18 isoenzymes identified to date. HDACs are classified into four groups (I -IV) based on their homology to yeast HDACs as summarized in Table 5[50,51].

The polycomb group proteins (PcG) repress gene activity by trimethylation of H3-K27 (histone 3 lysine 27) while TrxG proteins (Trithorax group) activate gene expression *via* H3-K4 histone trimethylation. The PcGs have 2 functions: polycomb repressive complex 1 (PRC1) maintains the silenced (H3-K27-me3, trimethylated) chromatin state and consists of CBX-2, 4, or 8 (chromobox homologue 2/4/8), PHC-1, 2, or 3 (polyhomeotic homologue 1/2/3), BMI1 (B-cell-specific Moloney murine leukemia virus integration site 1), and RING1A/B or RNF2 (RING finger domain protein). PRC2 initiates the repressive state by *de novo* trimethylation of H3-K27 and consists of EZH2 (enhancer of zeste homologue 2), SUZ12 (suppressor of zeste 12) and EED (embryonic ectoderm development)[39]. Together with other chromatin-modifying enzymes including DNMTs and HDACs,

the initially constituted suppression *via* H3K27-3me by PRC2 is maintained by PRC1 and allows fine-tuned, context-dependent regulation of gene silencing.

miRNAs are short (18-25 nucleotides), phylogenetically conserved single-stranded RNA molecules without protein-coding functions involved in the silencing of messenger RNAs (mRNAs)[52,53]. This post-transcriptional repression is accomplished by (partial) base-pairing with the respective mRNA causing either (1) inhibition of translation initiation; (2) inhibition of translation elongation; or (3) mRNA decay initiated by deadenylation of the mRNA following recruitment of a deadenylase complex[54,55]. The functions added a new layer of regulatory mechanisms affecting virtually all cellular functions[56,57]. The interaction between miRNAs and their target mRNAs ultimately leads to reduced levels of the regulated mRNA/protein. The current release of miRBase (v20[58]) lists 2578 mature miRNA sequences and it is estimated that over 60% of human mRNAs are direct miRNA targets[59]. miRNAs can function as either suppressors or oncogenes and their regulatory importance in human tumorigenesis has been demonstrated for various cancer types[60,61]. While miRNAs are *per se* categorized as an epigenetic mechanism, their cancer-related expression itself may be subject to epigenetic regulation by the above-mentioned mechanisms of chromatin modulation[62,63].

In the following paragraphs, we focus on their role in tumorigenesis in pancreatic cancer including their potential therapeutic exploitation by "epidrugs". For a general overview on cancer-related epigenetic mechanisms, we kindly refer the reader to recent comprehensive reviews: DNA methylation[64-66], histone (de)acetylation[67-70], histone methylation[71-73], and miRNAs[74-76].

Epigenetic mechanisms

DNA methylation in pancreatic cancer: As discussed in McCleary-Wheeler *et al*[8] DNA methylation may occur early during tumorigenesis of pancreatic cancer as some epigenetic alterations are already observed in the earliest lesion, *i.e.*, PanIN-1A, and their importance may increase during further tumor progression[30]. In line with these findings, mucin 4 (MUC4) gene expression is increased from normal to precancerous lesions to pancreatic cancer associated with an increasing frequency of MUC4 promoter hypomethylation[77]. Furthermore, not only DNA methylation but also hypomethylation and thus aberrantly high expression of a gene may represent an epigenetic feature in pancreatic carcinoma[78,79]. Table 3 summarizes the currently available literature on (deregulated) DNA methylation in pancreatic cancer.

Aberrant activation of developmental signalling pathways such as Hedgehog represents a frequently observed trait in human cancers[9]. He *et al*[80] have shown that the Hedgehog transcription factor Gli1 (GLI family zinc finger 1) targets epigenetic modifiers in pancreatic cancer, namely DNMT1 and DNMT3a. After showing a concomitantly higher expression of Gli1 and the DN-

MTs in pancreatic tumor samples, the authors proved by pharmacological inhibition using cyclopamine, Gli1 over-expression and siRNA (small interfering RNA)-based Gli1 knockdown, that the DNMT proteins are positive targets of this oncogenic pathway, suggesting a cross-talk between aberrantly activated embryogenesis pathways and activation of oncogenic epigenetic mechanisms in pancreatic cancer.

Related to the important role of epithelial-mesenchymal transition (EMT) in pancreatic tumor progression, FOXA1 and FOXA2 (forkhead box A1/2) transcriptions factor were identified as effective antagonists of EMT in pancreatic cancer due to their ability to positively regulate E-cadherin expression: in a study by Song *et al*[81], FOXA1/2 expression was lost during malignant progression and their promoter was extensively hypermethylated in a pancreatic cancer cell line. As the demethylation-mediated reactivation of E-cadherin required concomitant FOXA2 expression, the authors concluded that suppression of FOXA1/2 is necessary and sufficient for EMT in the progression of pancreatic cancer[81].

From the data summarized in Table 3 and the mentioned examples, it is clear that numerous genes and signaling targets are epigenetically regulated by means of DNA methylation in pancreatic cancer. Importantly, not only does late-stage, malignant pancreatic cancer display aberrant DNA methylation, but also earlier, pre-malignant lesions. It is important to note that DNA methylation may act in cooperation with other epigenetic mechanisms (histone modification) to achieve stable silencing of, for example, individual tumor suppressor genes. In a series of studies, Yamada *et al*[82-84] have shown that different mucin variants are regulated by different and complementary epigenetic mechanisms: MUC1, MUC2 and MUC5AC by DNA methylation and H3-K9 histone methylation. This fact has to be considered in therapeutic approaches which aim at reversing deregulated DNA methylation patterns, for example.

Histone-based epigenetics in pancreatic cancer: Acetylation and methylation of histones represent the 2 epigenetic mechanisms based on histone modifications for which currently data exist in the context of pancreatic tumorigenesis. Several studies have demonstrated the general relevance of HDAC enzymes in pancreatic cancer by showing that (1) HDAC2 is increased in pancreatic ductal adenocarcinoma, especially in poorly differentiated tumors[85]; and (2) the expression of HDAC7 is significantly increased in pancreatic adenocarcinoma samples; HDAC7 expression furthermore allows for discrimination between pancreatic adenocarcinoma from other pancreatic tumors (serous cystadenoma, IMPN)[86]. The potential of therapeutic targeting of HDACs in pancreatic cancers has been reviewed by Schneider *et al*[87]. As discussed in this section, several studies have unequivocally demonstrated the relevance of histone-based epigenetic mechanisms including the (oncogenic) functions of PRC1/2 protein complexes to contribute to pancreatic

tumorigenesis. These studies provided evidence of either altered/aberrant expression of these epigenetic regulators in pancreatic cancer samples or demonstrated the potential therapeutic exploitation of these mechanisms using cell-based *in vitro* studies.

An interesting cooperation between the ZEB1 (zinc finger E-box binding homeobox) transcription factor which is responsible for silencing of the E-cadherin gene (*CDH1*) and HDAC enzymes was investigated by Aghdassi *et al*[88]. In 25 surgical pancreatic cancer specimens, the authors found neither hypermethylation nor somatic mutations in the *CDH1* gene, but complexes of ZEB1/HDAC attached to the *CDH1* promoter. Knockdown of ZEB1 prevented this interaction resulting in histone acetylation and re-expression of E-cadherin. This study has provided additional insights into how EMT transcription factors cooperate with HDACs to silence E-cadherin, and thus promote EMT and tumor progression[89]. These data confirmed earlier results which demonstrated that downregulation of E-cadherin in metastatic pancreatic cancer cells is mediated by a repressor complex containing the EMT transcription factor Snail and the HDAC1 and HDAC2 enzymes[90].

The particular role of PRC proteins as epigenetic mechanisms has only recently become a focus in pancreatic cancer research. In several tumor types, overexpression of the *EZH2* gene is associated with poor prognosis, advanced stage, invasion and metastasis - for an overview see Crea *et al*[91]. As reviewed in Grzenda *et al*[39], *EZH2* overexpression promoted survival, angiogenesis, migration, proliferation and repression of E-cadherin. For pancreatic cancer, several studies demonstrated an involvement of components of PRC1/2 in malignant progression: Martínez-Romero *et al*[92] found the expression of the PRC1 proteins Bmi1 and Ring1b to be upregulated in PanIN lesions (Bmi1) and pancreatic adenocarcinoma (both components). These finding were confirmed by Song *et al*[93] showing that Bmi1 is correlated with lymph node metastasis and poor survival rates; furthermore, stable knockdown of Bmi1 reduced the levels of cyclin D1, CDK-2/4 (cyclin-dependent kinase), Bcl-2 (B-cell CLL/lymphoma 2) and phosphor-Akt while the expression of p21 and Bax was increased and associated with higher susceptibility towards apoptosis induction. Moreover, Wellner *et al*[94] showed that Bmi1 and other "stemness" factors were negative targets of the epithelial differentiation-linked miR-200c, -203 and -183 suggesting a possible mechanism for Bmi1 deregulation in pancreatic cancer. Another PRC1 member was investigated by Karamitopoulou *et al*[95]: CBX7 was analyzed in 210 ductal pancreatic adenocarcinomas and its expression was found to progressively decrease from normal pancreatic tissue, PanINs and invasive ductal adenocarcinoma-associated with increasing malignancy and a trend to shorter overall survival.

Using immunohistochemistry, Ougolkov *et al*[96] could show nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 68% (71/104) of pancreatic ad-

enocarcinomas and that its nuclear accumulation was more prevalent in poorly differentiated pancreatic adenocarcinomas (91%, 31/34). Using RNA interference, genetic depletion of EZH2 sensitized pancreatic cancer cells to chemotherapy and caused re-expression of p27 [cyclin-dependent kinase inhibitor 1B (p27, Kip1)] and decreased proliferation. Similar results were obtained by Toll *et al*[97] who demonstrated an inverse relationship between expression of EZH2 and E-cadherin in 54 pancreatic adenocarcinomas, and significantly longer survival in gemcitabine-treated patients with low expression of *EZH2*. Interestingly, inactivation of RUNX3 (runt-related transcription factor 3), a component of TGF-β signalling, is mediated by at least 2 epigenetic mechanisms, both EZH2[98] and DNA hypermethylation[99], highlighting their cooperation to convey a malignant phenotype in pancreatic cancer. Recently, miR-218 was demonstrated to be negatively regulated by EZH2 in pancreatic ductal adenocarcinoma[100]. MiR-218 was significantly reduced in primary tumor samples compared with normal adjacent tissue, and its silencing was mediated by EZH2 which binds to the miR-218 promoter, promotes formation of heterochromatin, and recruits DNMT-1, -3A and -3B. MiR-218 expression reduces proliferation *in vitro* and tumor formation as well as metastasis in nude mice[100]. The inverse regulatory relationship has also been recently observed between EZH2 and pre-miR101. Overexpression of pre-miR-101 reduced the binding of EZH2 to the promoter of the E-cadherin gene (*CDH1*) and increased the levels of E-cadherin[101].

Epigenetics in pancreatic cancer stem cells: An interesting and possibly therapeutically relevant aspect is the role of EZH2 in maintenance of stemness characteristics in cancer, particular its role in maintaining the self-renewal capabilities of cancer stem cells (CSC)[102-108]. This subpopulation of cancer cells has been characterized in pancreatic cancer by surface markers CD44, CD24, CD133, ESA (EpCAM, epithelial cell adhesion molecule)[109,110] and is thought to represent the population of cancer cells responsible for tumor maintenance, tumorigenicity, metastasis, and resistance to conventional chemotherapeutic drugs, as well as recurrence[109,111]. Similar to studies with CSC derived from hepatocellular carcinoma and acute myeloid leukemia[104,112], recent studies have demonstrated the therapeutic potential of epidrugs to directly target this tumorigenic subpopulation of pancreatic cancer cells: Avan *et al*[102] used an inhibitor of the EZH2 methyltransferase (DZNep, deazaneplanocin-A) and showed that treatment with DZNep reduced spheroid formation of pancreatic cancer cells and decreased the CD133+ subpopulation. Furthermore, the combination of DZNep and gemcitabine was shown to be highly synergistic and was accompanied by a reduced percentage of G2/M cells, reduced migration, increased E-cadherin expression and increased apoptosis[102]. The level of EZH2 has furthermore been suggested as an assay to effectively measure changes in the CSC subpopulation: us-

ing pancreatic and breast cancer cell lines, knockdown of EZH2 by RNA interference decreased the CSC subpopulation, confirming its role in CSC maintenance, and genes affected by EZH2 knockdown were inversely correlated with their expression in enriched CSC subpopulations[108]. The Hedgehog pathway has also been implicated in the maintenance of CSCs in various models[9,113]; interestingly a combination of Hedgehog inhibition (SANT-1) and SAHA (a pan-HDAC inhibitor; suberanilohydroxamic acid, Vorinostat) synergistically suppressed proliferation and colony formation in gemcitabine-resistant pancreatic adenocarcinoma cell lines by increased Bax expression, activation of caspase-3/7, increased p21 and p27 and reduced cyclin D1 expression. This study suggests that combined inhibition of stem cell-associated pathways (Hedgehog) and epigenetic drugs could be efficient in targeting the CSC subpopulation in pancreatic cancer[114]. A study by Nalls *et al*[115] could demonstrate that demethylating agents (5-aza-dC, 5-aza-2'-deoxycytidine) and the HDAC inhibitor SAHA restored the expression levels of miR-34a, which is reduced in pancreatic CSCs. These inhibitors caused a reduction in the EMT-related ZEB1, Snail, and Slug transcription factors, increased epithelial marker expression (E-cadherin) and, most importantly, reduced the number of viable pancreatic CSC, accompanied by reduced migration, colony formation and invasion of these cells.

Based on the above-mentioned functions and properties of CSC which is critical for tumor initiation, metastasis, progression and therapeutic resistance, these findings are of central importance and warrant further investigation to hopefully develop (epigenetics-based) therapeutic regimens specifically targeting this tumorigenic subpopulation in pancreatic cancer.

miRNA-based epigenetics in pancreatic cancer: Some reporters[116-119] and Park *et al*[120] have reviewed the available publications on differential miRNA expression in pancreatic cancer *vs* normal tissue culminating in a list of 64, partly overlapping individual miRNAs which were found to be deregulated in pancreatic cancer. Of these miRNAs, overexpression of miR-21, -155, -196a-2, -203, -210 and -222 was furthermore associated with poor outcome[120].

Table 4 provides an update (based on Park *et al*[120] 2011) of the currently available literature on the specific role of individual miRNAs in pancreatic cancer. All of these studies investigated the cellular/molecular mechanisms of the oncogenic or tumor-suppressive action of miRNAs, mainly by forced overexpression or knockdown of the respective miRNAs.

An example of how epigenetic mechanisms are employed to regulate the expression of tumor-suppressive miRNAs is shown in the study of Zhang *et al*[121]. From 12 miRNAs differentially expressed in pancreatic cancers *vs* adjacent normal tissue, miR-132 was downregulated in 16/20 pancreatic carcinomas accompanied by methylation of its promoter, as shown both in cell lines and tumor tissue. Sp-1 expression was correlated with miR-132

expression, and its binding affinity to the miR-132 promoter was significantly lower in pancreatic tumors relative to non-tumor samples.

As recently discussed[120,122], epigenetic features and especially miRNAs could also serve as biomarkers to allow specific and sensitive diagnosis of pancreatic cancer - an important approach as most patients with this disease remain without symptoms until the lesion has progressed to an advanced or metastatic stage. In this context, the use of miR-155 which showed upregulation in most IPMNs (83% of cases) has been analyzed in pancreatic juice by Habbe *et al*[123]. The authors confirmed upregulation of the miR-155 transcript in 60% (6/10) of IPMN-associated pancreatic juice samples but in none of the 5 control cases. Wang *et al*[124] profiled 4 miRNAs (miR-21, -210, -155, and -196a) in heparin-treated blood samples and found a sensitivity of 64% and a specificity of 89% to distinguish pancreatic cancer patients from healthy controls using this panel of miRNAs, thus proving the feasibility of plasma-based miRNA profiling as a potential biomarker for pancreatic cancer. Furthermore, Kawaguchi *et al*[125] investigated the utility of plasma miR-221 as a biomarker for cancer detection and monitoring tumor dynamics in 47 consecutive pancreatic cancer patients: similar to cancer tissue, plasma miR-221 levels were significantly higher in pancreatic cancer patients and correlated with distant metastasis and non-resectable status. Also, miR-21 serum levels were shown to be associated with overall survival of pancreatic cancer patients, and, in combination with 6 other miRNAs, allowed for correct classification of clinically suspected pancreatic cancer with a rate of 84%[126]. Similar results were obtained for miR-18a in plasma samples of patients with pancreatic cancer: miR-18a levels were significantly higher in 36 cancer patients compared with 30 healthy volunteers[127]. Kong *et al*[128] investigated the utility of several miRNAs as serum markers: while miR-21 distinguished pancreatic ductal adenocarcinoma patients from chronic pancreatitis and controls, miR-196a could distinguish resectable (stages Ⅰ and Ⅱ) and unresectable pancreatic ductal adenocarcinoma (Ⅲ and Ⅳ) as well as predict median survival time of pancreatic ductal adenocarcinoma patients (6.1 mo *vs* 12.0 mo for high *vs* low level miR-196a). Recently, miR-21 from pancreatic cyst fluid was investigated as a potential biomarker and could differentiate between benign, premalignant and malignant pancreatic cyst neoplasms[129].

POTENTIAL TARGETS FOR EPIGENETIC THERAPY IN PANCREATIC CANCER

The classic cancer progression model from PanIN to invasive carcinoma highlights genetic alterations in several oncogenes and tumor suppressor genes[130]. Hanahan *et al*[35,36] characterized additional distinct features of malignant tumor cells in their outstanding reviews on hallmarks of cancer that have also been identified in pancreatic cancer: sustaining proliferative signaling

(*e.g.*, activating mutations of K-ras[131]), evading growth suppressors (*e.g.*, deletions or mutations of *CDKN2A/p16*[Ink4A][132]), activating invasion and metastasis (*e.g.*, expression of CXCL12/CXCR4 [chemokine (C-X-C motif) ligand 12/(CXC receptor 4)[133]], enabling replicative immortality (*e.g.*, telomerase activation *via* loss of ATRX in pancreatic neuroendocrine tumors[134]), inducing angiogenesis [*e.g.*, increase in serum vascular endothelial growth factor (VEGF)[135]] and resisting cell death (*e.g.*, overexpression of anti-apoptotic Bcl-2[136]. Many of these alterations have been explored as targets for novel therapies (*e.g.*, anti-angiogenesis using the anti-VEGF antibody bevacizumab or anti-epidermal growth factor receptor directed therapies using erlotinib or cetuximab) achieved only marginal survival benefits in pancreatic cancer patients compared with standard therapy[137-139]. As outlined above, recent data also suggest strong roles for non-genetic events in pancreatic carcinogenesis and resistance to current therapies[8], *e.g.*, by modulating ABC drug transporters or interfering with cell death pathways (see Tables 2-4 for details).

Consequently, these regulatory mechanisms could represent interesting and potent novel targets for therapy to overcome resistance and to improve treatment outcome further[140,141]. Inhibitors of DNMT are nucleoside analogues of cytidine and currently azacytidine and decitabine are available for clinical use (Table 6), although the number of current trials is very limited. Zebularine is in preclinical development[142] with promising experimental data in pancreatic cancer[143].

Inhibitors of protein and histone deacetylases have been established as a novel approach to target hematologic and solid tumors[144]. Several phase I studies using the first-in-class molecule vorinostat (SAHA) are currently ongoing, especially in combination with cytotoxic agents or radiotherapy. Other agents like belinostat (PXD-101), entinostat (MS-275) or panobinostat (LBH-589) are at various stages of early clinical development, too, with progression-free survival or maximum tolerated dose as study endpoints. As described above, in addition to deacetylases, HATs can also regulate gene transcription. Here, curcumin (derived from the South Asian plant turmeric) has been demonstrated to effectively inhibit the activity of the HAT p300/CBP in cancer cells[145,146]. Although its pharmacokinetic properties are unsatisfying so far, it demonstrated early signs of clinical efficacy in pancreatic cancer patients in a phase Ⅱ setting[147].

Other epigenetic modifiers besides DNMT, HAT or HDAC have been identified and the first lead compounds are currently being extensively studied preclinically or are in early clinical phases. However, clinical data for pancreatic cancer is not available[148].

While miRNAs are considered useful tools for diagnosis, prognosis and possibly patient stratification[149], miRNA-based therapeutics are currently not available. Although preclinical data suggests that antagomiRs or miRNA replacement therapy is promising for pancreatic cancer models, clinical use is hampered by unresolved

Table 6 Trials using epigenetic agents in pancreatic cancer

Compound	Combination	Phase	Endpoint	ClinicalTrials.gov	Treatment
DNMT inhibitors					
Azacitidine	+ Gemcitabine	I	MTD	NCT01167816	
Azacitidine		II	PFS	NCT01845805	2
Decitabine			Various stages of development for solid tumors		3
Zebularine			Preclinical		
HDAC inhibitors					
Vorinostat	+ Marizomib	I	MTD	NCT00667082[234]	
Vorinostat	+ Radiation	I / II	MTD, PFS	NCT00948688	
	+ 5-FU				
Vorinostat	+ Radiation	I	MTD	NCT00983268	
	+ Capecitabine				
Vorinostat	+ Radiation	I / II	MTD	NCT00831493	
Belinostat			Various stages of development for solid tumors		
Entinostat		I	MTD	NCT00020579[235]	
Entinostat	13-*cis* retinoic acid	I	MTD		
Panobinostat	+ Bortezomib	II	PFS	NCT01056601[236]	1
HAT inhibitors					
Curcumin		II	Survival	NCT00094445[147]	1
Curcumin	+ Gemcitabine	II	TTP	NCT00192842	1
Curcumin	+ Gemcitabine	III		NCT00486460	1
	+ Celecoxib				
Curcumin		I	MTD	-[237]	
Curcumin	+ Gemcitabine	I	MTD	-[238]	
Curcumin	+ Gemcitabine	I / II	MTD	-[239]	

1: Palliative; 2: Postoperative adjuvant; 3: Both. MTD: Maximum tolerated dose; PFS: Progression-free survival; HDAC: Histone deacetylase; HAT: Histone acetyltransferase.

drug delivery and the fact that one miRNA also has several target mRNAs, thus possibly being too unspecific[150,151].

Overall, as most of the agents highlighted above are currently in early phases of clinical development, no clear data on efficacy of epigenetic agents in pancreatic cancer are available, but promising preclinical[152] and early clinical data warrant further development.

CONCLUSION

Due to the poor prognosis of pancreatic cancer patients, understanding the molecular events driving this devastating tumor disease is central for development of alternative and more effective treatment strategies and for the determination of reliable diagnostic markers. Recent research on epigenetic mechanisms has greatly enriched our knowledge about the regulatory traits involved in initiation, progression and metastasis of pancreatic cancer. As reviewed in this article, DNA-, histone- and miRNA-based epigenetic events have been demonstrated to play a role in pancreatic cancer and could serve as future therapeutic targets aiming at reversing the epigenetic deregulation of the cellular machinery. Initial clinical trials at stages I - III using inhibitors of DNMTs, HDACs and HATs are currently under way and open the door to development of novel and hopefully more effective 'epidrugs' for patients with pancreatic cancer.

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2014 ADVANCES IN PANCREATIC CANCER

Personalising pancreas cancer treatment: When tissue is the issue

Katrin M Sjoquist, Venessa T Chin, Lorraine A Chantrill, Chelsie O'Connor, Chris Hemmings, David K Chang, Angela Chou, Marina Pajic, Amber L Johns, Adnan M Nagrial, Andrew V Biankin, Desmond Yip

Katrin M Sjoquist, NHMRC Clinical Trials Centre, University of Sydney, Sydney NSW 1450, Australia

Katrin M Sjoquist, Cancer Care Centre, St George Hospital, Kogarah NSW 2217, Australia

Venessa T Chin, Lorraine A Chantrill, Chelsie O'Connor, David K Chang, Angela Chou, Marina Pajic, Amber L Johns, Adnan M Nagrial, Andrew V Biankin, The Kinghorn Cancer Centre, and the Cancer Research Program, Garvan Institute of Medical Research, Sydney NSW 2010, Australia

Lorraine A Chantrill, Macarthur Cancer Therapy Centre, Campbelltown Hospital, Campbelltown NSW 2560, Australia

Lorraine A Chantrill, David K Chang, Marina Pajic, St Vincents Hospital Clinical School, Sydney UNSW 2010, Australia

Chelsie O'Connor, Cancer Therapy Centre, Liverpool Hospital, Liverpool NSW 2170, Australia

Chris Hemmings, St John of God Pathology, Subiaco WA 6904, Australia

David K Chang, Andrew V Biankin, Department of Surgery, Bankstown Hospital, Eldridge Road, Bankstown, Sydney NSW 2200, Australia

David K Chang, Andrew V Biankin, Faculty of Medicine, South Western Sydney Clinical School, University of NSW, Liverpool NSW 2170, Australia

David K Chang, Andrew V Biankin, Wolfson Wohl Cancer Research Centre, Institute of Cancer Sciences, University of Glasgow, Glasgow G61 1BD, United Kingdom

Angela Chou, Department of Anatomical Pathology, St. Vincent's Hospital, Darlinghurst, Sydney NSW 2010, Australia

Andrew V Biankin, West of Scotland Pancreatic Unit, Glasgow Royal Infirmary, Glasgow G4 0SF, United Kingdom

Desmond Yip, Department of Medical Oncology, The Canberra Hospital, Garran ACT 2605, Australia

Desmond Yip, ANU Medical School, Australian National University, Acton ACT 2601, Australia

Author contributions: Sjoquist KM, Chantrill LA and Yip D contributed to Conception and design; all authors contributed to drafting the article or revising it critically for important intellectual content, and final approval of the version to be published.

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Correspondence to: Desmond Yip, Associate Professor, **Clinical Director,** Department of Medical Oncology, The Can-

berra Hospital, Yamba Drive, Garran ACT 2605, Australia. desmond.yip@anu.edu.au
Telephone: +612-6244-2220 **Fax:** +612-6244-4266
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Abstract

The treatment of advanced pancreatic cancer has not moved much beyond single agent gemcitabine until recently when protocols such as FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin) and nab-paclitaxel-gemcitabine have demonstrated some improved outcomes. Advances in technology especially in massively parallel genome sequencing has progressed our understanding of the biology of pancreatic cancer especially the candidate signalling pathways that are involved in tumourogenesis and disease course. This has allowed identification of potentially actionable mutations that may be targeted by new biological agents. The heterogeneity of pancreatic cancer makes tumour tissue collection important with the aim of being able to personalise therapies for the individual as opposed to a one size fits all approach to treatment of the condition. This paper reviews the developments in this area of translational research and the ongoing clinical studies that will attempt to move this into the everyday oncology practice.

Key words: Pancreatic neoplasms; Molecular targeted therapy; Genomics; Tissue banks; Chemotherapy

Core tip: State of art review of genomic developments in pancreatic cancer that will hopefully lead to a new treatment paradigm of recognising that pancreatic cancer is a heterogenous disease. Adequate tissue col-

lection is important to allow biomarker testing and molecular sequencing to allow determination of actionable mutations so that personalised therapies can be used in a rational manner.

INTRODUCTION

Until recently, progress in understanding of pancreas cancer has been frustratingly slow. Prognosis remains exceedingly poor, with the majority of patients presenting with rapidly lethal advanced disease[1,2]. Distinct phenotypes, while clinically recognised, have been difficult to capture using common diagnostic tests. In addition, the value of doing so for directing therapy has been minimal, with limited treatment options and a lack of alternatives to gemcitabine which has remained the standard of care for advanced disease until recently.

Advances in technology have recently accelerated our understanding of the biology of pancreatic cancer and tumour-host interactions. Recent initiatives such as Australian Pancreatic Cancer Genome Initiative (APGI, http://www.pancreaticcancer.net.au/apgi) and International Cancer Genome Consortium (ICGC, http://icgc.org) have seen major progress in the acquisition of high quality biospecimens for molecular studies in comprehensive cancer cohorts. Whole genome sequencing has facilitated identification of potentially actionable mutations with greater sensitivity and specificity. As tissue requirements and costs for genome sequencing decrease, the potential to select treatments in a "personalised" manner based on tumour biology moves closer to the clinic[3].

The move towards "personalised" treatment of pancreatic cancer is not without challenges. The anatomical location of the pancreas and clinical presentation of the majority of cancers in advanced stages present particular barriers to diagnostic and exploratory tissue sampling. The relative inaccessibility of the pancreas, compared to many other tumour types, limits the ability to collect adequate µ-tissue (for example core biopsies) from primary lesions. Recent trials have demonstrated that core biopsies are currently feasible in some settings; the Liberal Education and America's Promise (LEAP) trial required core biopsy for trial participation and were able to do this prior to enrolment in 367 participants with metastatic disease[4]. Patients presenting *de novo* with advanced disease can rapidly deteriorate and any additional diagnostic tests need to demonstrate therapeutic value and provide useful information with minimal delay to be useful in routine practice.

Tumour and patient profiling are critical in understanding the disease, developing new treatments, and better selecting patients for existing treatments. The timely, accurate and appropriate collection of tissue and blood samples are fundamental to driving future research and evolving patient care in the era of personalised and precision medicine. Future strategies, including profiling of circulating tumour DNA[5], may minimise the invasiveness of testing but at present access to tumour tissue remains important in developing new treatment strategies and understanding their failures.

This review highlights recent advances in understanding of pancreas cancer at a molecular level including key signalling pathways and markers of treatment sensitivity. The current evidence base for a personalised approach is summarised, together with relevant ongoing trials.

RECENT ADVANCES IN SYSTEMIC THERAPY FOR ADVANCED PANCREATIC CANCER

Very little progress has been made in the systemic treatment of advanced pancreatic cancer until recent years. Gemcitabine a nucleoside analogue became established as the standard therapy following the demonstration of improved survival and clinical benefit (pain, performance status and weight) against 5-fluorouracil[6]. This led to the subsequent focus on combining other drugs with gemcitabine to test doublets against gemcitabine monotherapy. For a time no doublet was clearly superior to monotherapy. A number of meta-analysis of gemcitabine combination studies have been carried out[7-9]. These have shown an improvement in survival with platinum based combinations as well as fluorouracil based combinations[10]. There was a suggestion of more benefit from combination therapy in good performance status patients and a worse prognosis in poor performance patients with combination therapy[8]. The most recent meta-analysis of 26 studies with a total of 8808 patients has found that the relative risk of 1 year survival was lower for monotherapy when compared to combinations with platinum, fluoropyrimidine and targeted agents respectively but no statistical differences were found[9]. When median progression free survival and overall survival were assessed only fluoropyrimidine was statistically superior. The combination therapies were associated with more toxicity.

A non-gemcitabine based intensive chemotherapy schedule FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin) in good performance status patients under 76 years of age has shown clear superiority to gemcitabine monotherapy (response rate 31.6% *vs* 9.4% (*P* < 0.001), median survival 11.1 mo *vs* 6.8 mo (*P* < 0.01) and one year survival 48.4% *vs* 20.6% at a cost of increased toxicity[11]. Quality of life was assessed in the study and it was found that the FOLFIRINOX arm improved global health status and the time until definitive

deterioration was significantly longer than gemcitabine[12].

A recently reported phase III trial demonstrated for the first time an overall survival benefit with gemcitabine based doublet therapy. The MPACT trial randomised gemcitabine *vs* gemcitabine plus nab-paclitaxel (Abraxane) found an improvement in median survival from 6.7 to 8.5 mo with 1 year survivals of 22% and 35% respectively ($P < 0.001$)[13]. The response rate also increased in the combination arm (7% *vs* 23%, $P > 0.001$), although this was at the expense of higher rates of myelosupression and peripheral neuropathy with the doublet.

The role of biological agents has been studied in pancreatic cancer. They have generally been tested in combination with the traditional chemotherapy backbone of gemcitabine. One positive trial was the combination of gemcitabine with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib *vs* gemcitabine alone[14]. This NCIC phase III study found no difference in response rates or quality of life between the arms but did find an improvement of median overall survival from 5.91 to 6.24 mo ($P = 0.038$) with the addition of erlotinib and the one year survival improved from 17% to 23% ($P = 0.023$)[14]. The very modest benefit in median survival (2 wk) raises the question as to whether this is clinically relevant although there is a tail of increased survivors at one year. Tumour tissue was collected in this study from 184 patients out of 569 patients with only 162 with sufficient tumour for immunohistochemistry, limiting the power to detect whether EGFR expression had any effect on outcome. Although a positive relationship between the development of rash and survival was observed in this study suggesting that this may be a identify a favourable prognostic subgroup, a subsequent study that explored dose escalation of erlotinib until development of rash found no added benefit to standard fixed dose erlotinib when combined with gemcitabine[15]. Also of note, a subsequent SWOG phase III trial using the monoclonal antibody cetuximab as an anti-EGFR strategy in advanced pancreatic cancer found no additional benefit when added to gemcitabine[16].

Antiangiogenics have been tested in a number of phase III trials in combination with gemcitabine. These include the anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibodies bevacizumab[17,18] and aflibercept[19] as well as the oral small molecule tyrosine kinase inhibitor axitinib[20,21]. These trials have been uniformly negative and suggest that targeting VEGF is an ineffective strategy in pancreatic cancer. Similarly, trials of the matrix metalloproteinase inhibitors marimastat[22] and BAY 12-9566[23] have also been negative. A phase III trial of the multikinase inhibitor sorafenib in combination with gemcitabine was also negative[24], as was a study of enzastaurin a PKCbeta and PI3K/AKT signalling inhibitor[25].

These trials have all taken a "one size fits all" approach to treatment of advanced pancreatic cancer in enrolling unselected patients. With the recognition that pancreatic cancer is a biologically heterogenous disease, a person-alised approach would mean selecting out patients into enriched groups with biomarker or genomic profiles of activated pathways that are more likely to respond to targeted agents being tested therapeutically[26]. This approach has been taken with the randomised phase II RECAP trial (NCT01423604) of capecitabine in combination with placebo or ruxolitinib a JAK1 and JAK2 inhibitor in which patients with recurrent or treatment refractory pancreatic cancer has analysed a prespecified subgroup of patients identified prospectively as likely to benefit from JAK inhibition. Within this subgroup which is half of the randomized population the hazard ratio for survival was 0.47 with ruzolitinib with 6 mo survival being 42% and 11% for placebo[27]. This trial is yet to be formally reported but a phase III study is expected to be launched soon.

Current understanding of core signalling pathways in pancreatic cancer

Detailed molecular analysis of pancreatic cancer began at the beginning of the 21st century, with *CDKN2A*, *SMAD4*, *TP53* and *KRAS* the first candidate genes identified[28-32]. In late 2008 Jones *et al*[33] published a seminal paper in Science detailing global genomic analysis of 24 pancreatic cancers. In this paper, the authors made the case for 12 core signalling pathways that are genetically altered in the majority of pancreas cancers.

One pathway they highlighted was Wnt/Notch and Hedgehog signalling. Four years later, thanks to the international genome sequencing efforts described above, Biankin *et al*[34] published genomic data from 142 early stage pancreatic cancers. Although substantial heterogeneity was identified, 16 genes were significantly mutated. Reassuringly, some of these were common to those identified by Jones *et al*[33] but additional novel mutated genes were identified. Of these, the strongest signal was obtained from the SLIT/ROBO pathway of axon guidance that was identified previously in 2003[35,36]. Further work on this pathway to establish its role in tumourigenesis of pancreatic cancer is ongoing. Clearly the ongoing challenge for biologists in this field is to determine drivers of pancreas cancer, understanding that there may be different drivers in different cases. Using this method of separating pancreatic cancers into subgroups by driver has led us to test targeted, personalised treatment in animal models and also in human subjects.

Potential "actionable mutations" based on molecular profiling of pancreatic cancers

Several actionable changes have been identified in pancreas cancer; those with greatest potential clinical significance are summarised in Table 1.

Some of these were able to be verified with accredited, confirmatory genomic tests that are available commercially, such as *KRAS* mutation testing, *BRCA1* and *BRCA2* mutation testing and *ATM* mutation testing. It is envisaged that the future will see targeted sequencing panels for pancreas cancer being commercially available, accredited and clinically applicable with short timelines

Table 1 Potentially ''actionable'' phenotypes and supporting evidence

Actionable phenotype	Therapeutic	Rationale	Molecular characterization
Gemcitabine responsive	Gemcitabine	In PC, Phase Ⅲ trials showed benefit in adjuvant (DFS 13.4 mo *vs* 6.9 mo) and palliative setting (MS 5.65 *vs* 4.41)[6,87]	High hENT1, hCNT1, hCNT3 Phase Ⅲ data suggested that hENT1 correlated with response to Gemcitabine in adjuvant setting in PC[65,88], however this was not shown in the metastatic population[89]
Anti-EGFR responsive	Erlotinib, Cetuximab	A Phase Ⅲ trial showed that Erlotinib plus Gemcitabine had an overall survival benefit (HR = 0.82) compared with Gemcitabine alone in PC[14] Phase Ⅲ data did not show a difference in OS when Cetuximab was added to Gemcitabine in an unselected population with PC[16]	Classical subtype PC cell lines with a "classical" subtype were shown to be more sensitive to Erlotinib[90] EGFR expression did not correlate with response to Cetuximab in patients with PC[16]
Taxane responsive	*nab*-Paclitaxel	Phase Ⅲ data showed that the addition of *nab*-Paclitaxel to Gemcitabine increased PFS (HR = 0.69) and OS (HR = 0.72) in the metastatic PC population[13]	SPARC expression (stromal) A phase Ⅰ/Ⅱ study showed that SPARC expression in the stroma correlated with survival[71]
5-FU responsive	5-Fluorouracil	Small phase Ⅲ trials showed activity of 5-FU containing regimens in the metastatic population in PC[91,92] 5-FU was shown to prolong survival when used in the adjuvant setting in PC (HR = 0.7)[93]	Thymidylate Synthase High intra-tumoural expression was shown to correlate with an increased benefit from 5-FU based chemotherapy in pre-clinical[94] and retrospective patient populations[95]
Irinotecan responsive	Irinotecan	In PC, a small effect as monotherapy has been shown in the second-line setting[96], and a significant effect on OS was shown when used as part of FOLFIRINOX (HR = 0.57)[11]	Topoisomerase 1 expression High topo 1 expression was associated with a larger benefit from Irinotecan containing regimens in metastatic colorectal cancer[97]
HER2 amplified	Trastuzumab	Has shown activity in HER-2 overexpressing breast and gastric cancers[39,98,99] Phase Ⅱ trials do not show a benefit of adding Trastuzumab to Gemcitabine in PC (OS = 7 mo), however, no standardised approach to FISH testing was used[41,70]	*HER2* amplification Pre-clinical studies suggested that *HER2* overexpression predicts a response to Trastuzumab in PC[100]
m-TOR responsive	Everolimus, Temsirolimus	A phase Ⅲ trial of Everolimus in renal cell cancer shows prolongation in PFS (PFS 4 mo *vs* 1.9 mo)[101] Phase Ⅱ data showed minimal activity (OS = 4.5 mo) of Everolimus for second line treatment in an unselected population of patients with metastatic PC[102] A case study in a patient with PC and Peutz-Jeghers syndrome (SK11 deficient) responds to Everolimus[103]	P-TEN Deficient, High p-mTOR/p70S6, AKT amplified, STK11/LKB1 deficiency, PI3K mutation Pre clinical studies showed that p-TEN deficient cell lines are sensitive to m-TOR inhibitors[104] Retrospective studies suggested that SK11, p-MTOR, p-70S6, PI3K and AKT can select tumours that will respond to m-TOR inhibitors[103,104-110]
VEGF inhibitor responsive	Sunitinib, Bevacizumab	Phase Ⅲ trial showed no benefit with adding Bevacizumab to Gemcitabine in an unselected population of patients with PC[17] Phase Ⅱ data showed that maintenance Sunitinib after primary chemotherapy improved 2 yr OS (22.9% *vs* 7%) in the metastatic PC population[109]	CSF1R up-regulation, High HIF-α expression *In vitro* studies showed CSF-1R up-regulation was associated with response to Sunitinib in AML[110] High HIF-α predicted response to Sunitinib in a retrospective cohort in renal cell cancer[111]
DNA damage repair deficient	Platinum; MMC; PARP inhibitor	*In vitro* work showed that cells with defects in BRCA2 are preferentially sensitive to PARP inhibitors[112] Case reports of PC patients with BRCA2 deficient tumours respond to PARP[113,114] Multiple clinical trials are on-going assessing the effects of PARP inhibition	DDR signature; mutation of DDR genes, *BRCA/ATM/PALB2* Loss of BRCA1 was associated with sensitivity to DNA damaging agents[115] BRCA2 mutations were associated with improved response to platinum agents[116] *In vivo* studies showed PALB2 inactivation was a determinant of response to DNA damaging agents in PC[114]
SMO inhibitor responsive	Saridegib, Vismodegib	A phase Ⅱ trial found that Saridegib plus Gemcitabine was no better than Gemcitabine alone in an unselected population of metastatic PC patients (data not published)[117] *In vivo* studies show SMO inhibitors block metastasis formation in pancreatic cancer[118]	Gli1 and PTCH1 transcript levels GLI1 mRNA may predict response to SMO inhibitors in pancreatic cancer *in vivo*[119] High Gli1/PTCH1 transcript levels were correlated with response to SMO inhibitors in CLL[120]

PC: Pancreatic cancers; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; DFS: Disease-free survival; OS: Overall survival.

and low cost. Others, such as Her2 upregulation, can be tested for using immunohistochemistry and *in situ* hybridisation.

Her2 as an example of an "actionable" mutation

The *HER2* (human epidermal growth factor receptor)/neu/*ERBB2* gene is a member of a family of genes that

play a role in regulating cell growth. *HER2* signalling promotes cell proliferation through the RAS-MAPK pathway and inhibits cell death through the phosphatidylinositol 3'-kinase-AKT-mammalian target of rapamycin (mTOR) pathway. Although *HER2* overexpression has been described in a variety of human malignant conditions, gene amplification is uncommon except in breast and gastric cancer[37]. Anti-*HER2* therapy is clinical indicated and effective for both *HER2* amplified breast[38] and gastric[39] cancers. There is growing evidence that *HER2* is an important biomarker and key driver of tumourigenesis in pancreatic cancer.

Recent evidence suggests *HER2* amplification occurs in 2% of pancreatic ductal adenocarcinoma, and may potentially respond to anti-HER2 therapy[37], similar to *HER2*-amplified breast cancer. On a molecular level, *HER2*-amplified pancreatic cancers demonstrated a mRNA expression profile which clustered with the HER2-amplified intrinsic subtype of breast cancer using the PAM50 classifier. Clinically, *HER2*-amplified pancreatic cancers showed an atypical metastatic pattern characterized by spread to the lungs and brain with avoidance of the liver, not unlike the pattern of spread seen in *HER2*-amplified breast cancer. These findings suggest that *HER2* is likely to be the main driver of tumorigenesis in this subgroup of pancreatic cancer, analogous to *HER2*-amplified breast cancer and may respond to anti-*HER2* therapy.

Three clinical trials have assessed anti-Her2 therapy in pancreatic cancer. All 3 were single arm phase Ⅱ trials utilising anti-Her2 agents active in other cancers in conjunction with traditional cytotoxics. Only 2 of the trials selected patients based on Her2 status, and utilised immunohistochemistry alone to detect *HER2* overexpression[40-42].

In Safran's first study patients with *HER2* overexpressing metastatic pancreatic cancers were recruited and showed a response rate of only 6% which was considered as not significantly different from historical controls of gemcitabine alone[40]. The majority of the patients recruited however had HER2 2+ tumors. In Safran's second study lapatinib (a dual *HER1* and *HER2* inhibitor) and gemcitabine were given to an unselected population of patients with metastatic pancreatic cancer[42]. The study was terminated after 6 mo due to poor response rate. Harder *et al*[41] recruited 17 patients with HER2 expressing metastatic pancreatic cancer for trastuzumab and capecitabine, and this study closed prematurely due to lower than expected prevalence of HER2 3+ tumours and therefore slow accrual.

The selection of patients was based on HER2 expression using immunohistochemstry alone and these were not standardized assays performed in reference laboratories. As a result it is possible that the use of non-standardised assys performed outside accredited reference laboratories overestimated HER2 positivity. The likely overestimation of HER2 positivity underpowered the trials and makes a negative result difficult to interpret.

Identifying HER2 overexpressing pancreatic cancers

(PC) by genomic profiling has the potential to identify a cohort more likely to benefit from anti-HER2 therapy. This enrichment strategy is being utilised in the recently opened IMPaCT (Individualised Molecular Pancreatic Cancer Therapy) trial (Table 2) of which several authors are investigators.

Preclinical trials of repurposed drugs in patient-derived xenografts

In order to maximize benefit to patients clinical trials should be conducted in populations based on molecular characteristics[43]. This highlights the importance of biomarker driven therapeutic development. Such trials are expensive, labour intensive and pose significant logistical difficulties which in PC, are compounded by the rapidity of clinical deterioration and the small percentage of patients who are well enough to receive more than one line of chemotherapy. Using patient derived xenografts presents an attractive option to test potential biomarkers and partnered therapeutic interventions.

Xenograft models derived from established tumour cell lines may not fully recapitulate the complexities of human disease and therefore may not be the ideal medium with which to test novel therapeutics[44-46]. In addition the vast majority of cell lines that have been used in the past do not have associated germline sequence data. As a consequence, the accuracy of genomic aberrations identified by comparing to a reference sequence is not sufficient for subsequent testing of genotype-guided treatment strategies. Genetically engineered mouse models (GEMM) will develop PC predictably and can be used to study pancreatic carcinogenesis[47]. However, Singh *et al*[48] showed that the PDAC *Pdx1-Cre LSL-Kras*^{G12D}*p16/p19*$^{fl/fl}$ GEMM had a greater response to gemcitabine than typically observed in the patient population, suggesting these models too lack the heterogeneity and complexity of the human condition.

Primary xenografts are generated directly from engraftment of individual human tumour tissue into severely immunocompromised mice [nonobese diabetic /severe combined immunodeficiencies IL2rg -/- (NSG) mice] allowing efficient engraftment of the tumour[49,50]. These have been shown to faithfully represent the histopathological, biological and genomic characteristics of the primary tumour[51,52]. These models may represent valuable tools for testing novel therapies. Primary xenograft models have been used to test novel therapies in childhood leukaemia[53-55] and neuroblastoma[56,57]. More recently in PC, primary xenografts have been used to test the efficacy of sorafenib and everolimus alone and in combination[58].

The generation of primary xenografts provides a renewable and valuable resource with which multiple treatments may be studied. Large pre-clinical trials may be designed where a specific tumour of interest may be examined for its sensitivity to numerous different therapies or efficacy of a single novel therapy may be examined in a range of tumours with different molecular profiles. The

Table 2 Ongoing biomarker directed therapy trials in pancreatic carcinoma

Trial identifier[1]	Name of study	Phase	Sponsor	Arms	Primary outcome	Biomarker
ACTRN1261200077897	The IMPACT trial: Individualised Molecular Pancreatic Cancer Therapy A randomised open label phase II study of standard first line treatment or personalised treatment in patients with recurrent or metastatic pancreatic cancer	II	The Australasian Gastro-Intestinal Trials Group Collaborating groups: Australian Pancreatic Cancer Genome Initiative Sydney Catalyst; the Translational Cancer Research Centre of Central Sydney and Regional NSW	Patients with actionable phenotypes randomised 1:1 to Standard -gemcitabine alone OR Personalised Treatment -allocated based on molecular phenotype: HER2 positive sub-group - gemcitabine plus trastuzumab Homologous recombinant defects subgroup: 5FU plus MMC antiEGFR responsive sub-group: gemcitabine plus erlotinib	Progression free survival (initial pilot phase will assess feasibility of personalised approach)	Identification of actionable phenotypes based on molecular phenotype in tumour tissue in one of 3 subgroups: HER2 positive (HER2/neu amplification) subgroup Homologous recombination defects (BRCA1, BRCA2 or PALB2 mutation) AntiEGFR phenotype subgroup (KRAS wildtype or KRAS codon 13 mutation)
NCT01188109	Gemcitabine/cisplatin for resected pancreas cancer: Establishing the role of ERCC1 in treatment decision	II	Emory University	Gemcitabine and cisplatin	Progression free survival and overall survival	Immunohistochemistry, rt-PCR, and single nucleotide polymorphism assessment to determine status of ERCC1 expression and gene
NCT01488552	A Phase II study of induction consolidation and maintenance approach for patients with advanced pancreatic cancer	I / II	Pancreatic Cancer Research Team	Gemcitabine + Nab-paclitaxel induction FOLFIRINOX consolidative Metformin + targeted agent selected by biomarkers for maintenance	Complete response rate	IHC Analysis will be performed on a fresh tissue biopsy of the tumor after chemotherapy has been administered. A targeted-based regimen will be determined from the results of the IHC analysis for the next therapy given to the patient in the maintenance phase of the study
NCT01524575	Gemcitabine and oxaliplatin in the management of metastatic pancreatic cancers with low expression of ERCC1	Phase II	University of Hawaii	Gemcitabine+oxaliplatin	6 mo overall survival	Low expression of ERCC1 protein and mRNA expression
NCT01888978	A Pilot Study of Molecularly Tailored Therapy for Patients With Metastatic Pancreatic Cancer	Phase II	Georgetown University	Gemcitabine+oxaliplatin Gemcitabine + 5FU Gemcitabine + docetaxel FOLFOX6 Oxaliplatin + docetaxel FOLFIRI Docetaxel-irinotecan	Timing of biopsy and treatment Number of days from study entry to biopsy to molecular results to first dose	Selection of doublet treatment on basis of molecular analysis of tumour
NCT01585805	Gemcitabine Hydrochloride and Cisplatin With or Without Veliparib or Veliparib Alone in Patients With Locally Advanced or Metastatic Pancreatic Cancer	Randomised phase II	National Cancer Institute	Cisplatin+gemcitabine +/-veliparib	Optimal dose of veliparib Response rate	BRCA1 or BRCA2 mutation carrier
NCT01586611	Study of Gemcitabine *vs* FOLFOX in the First Line Setting for Metastatic Pancreatic Cancer Patients Using Human Equilibrative Nucleoside Transporter 1 (hENT1) Biomarker Testing	Phase III	AHS Cancer Control Alberta	FOLFOX Gemcitabine	PFS between arms in hENT1 high and hENT1 low patients	hENT1

NCT01454180	Study of Individualized Selection of Chemotherapy in Patients With Advanced Pancreatic Carcinoma According to Therapeutic Targets	Phase II	Centro Nacional de Investigaciones Oncologicas CARLOS III	Arm A: Physician discretion; Arm B: Therapeutic target guided: Gemcitabine; Gemcitabine + capecitabine; Gemcitabine + erlotinib; FOLFOXIRI; FOLFOX; FOLFIRI	Overall survival	Therapeutic targets expressed in tumour tissue
NCT01726582	Molecular Profiling to Guide Neoadjuvant Therapy for Resectable and Borderline Resectable Adenocarcinoma of the Pancreas	Phase II	Medical College of Wisconsin	Targeted chemotherapy prior to surgery; Standard FOLFIRINOX chemotherapy prior to surgery; Gemcitabine after surgery; No additional therapy after surgery; Targeted chemotherapy after surgery; Chemoradiotherapy (Targeted chemotherapy include the following schedules: FOLFIRINOX, FOLFIRI, gemcitabine+irinotecan, gemcitabine + oxaliplatin, gemcitabine + cisplatin, gemcitabine+nab-paclitaxel, capecitabine + nab-paclitaxel, gemcitabine, capecitabine, 5FU)	Resectability rate	Molecular profile of tumour will point to particular chemotherapy treatment

Trial number prefixes correspond to location of registry/portal. ANZCTRN: Australian New Zealand Clinical Trials Registry; 5FU: 5-fluorouracil; FOLFIRINOX: Fluorouracil, leucovorin, irinotecan and oxaliplatin; MMC: Mitomycin C; IHC: Immunohistochemistry; ERCC1: Excision repair cross complementation gene-1.

impact of this is two-fold, with the opportunity for a patient's tumour to undergo pre-clinical testing to determine the clinician's choice of therapy having obvious advantages to patient outcomes but also, that tumours bearing particular biomarkers of interest may be tested extensively against existing and novel therapies to guide the design of molecularly driven human clinical trials.

PREDICTING RESPONSE TO TREATMENT/IMPROVING TREATMENT DELIVERY

Another important facet of personalising therapy for pancreatic cancer is identifying patients who will derive net clinical benefit from existing treatments. Biomarkers of potential benefit or toxicity from existing systemic therapies as well as radiation therapy have been identified.

Identifying biomarkers for patients likely to benefit from radiotherapy treatment

Autopsy studies[59,60] have shown that more than 80% of patients with pancreatic cancer develop distant metastases even in those who have undergone curative resection, suggesting the presence of occult metastases at time of surgery. Therefore, patients who would benefit from local radiotherapy treatment are those less likely to develop systemic disease. Futures areas of research should not only focus on identifying those patients with locally aggressive disease but also those with radiosensitive tumours more likely to respond from radiotherapy treatment.

An autopsy study[61] found that positive staining for the intracellular protein DPC4 (or SMAD4) might indicate a patient that was more likely to harbour locally aggressive disease. Only twenty-two percent of patients with no metastatic disease at autopsy showed loss of expression of DPC4. Conversely, 73% of patients with extensive metastatic disease demonstrated loss of expression of this protein.

Identification of a biomarker of radiosensitivity has been explored in other tumour sites. The XRCC1 (X-ray repair cross-complementing group 1) protein is involved in base excision repair. A single nucleotide polymorphism known as Arg399Gln has been shown to affect radiosensitivity. The ECOG 1201 Phase Ⅱ trial analysed patients to determine whether the presence of this allele affected complete response rates after neoadjuvant cisplatin based chemoradiotherapy in oesophageal adenocarcinoma[62]. Fifty-two percent of patients had the Arg399Gln allele and only 6% of those had a complete response at time of surgery 5 wk after completion of their neoadjuvant treatment. The odds ratio for failing to undergo a complete pathological response in the presence of this allele was 5.37 (P = 0.062). This did not translate to a reduction in disease free or overall survival though it does suggest there are certain patients who are more likely to respond to radiotherapy.

Optimising drug delivery with predictive biomarkers

The co-development of novel chemotherapeutic and therapeutic strategy with companion diagnostics is the paradigm of modern clinical oncology. Outcomes from these efforts have been somewhat mixed to date, and the reasons are many and complex. For the purpose of this review, the authors will only concentrate on two drugs that have been approved for use in pancreatic cancer.

Gemcitabine: The putative biomarkers of gemcitabine responsiveness include nucleoside transporters such as hENT1, hCNT1/3 and kinases involved in gemcitabine metabolism such as deoxycytidine kinase[63,64]. The most studied biomarker of therapeutic responsiveness to date in PC is hENT1, a membranous equilibrative nucleoside transporter encoded by the *SLC29A1* gene. There is promising evidence to support the role of hENT1 in gemcitabine responsiveness in PC cells both *in vitro* and *In vivo*, but its precise role as a predictive biomarker in the clinic has not been well established, with conflicting results reported. Small cohort studies and retrospective analysis of large Phase Ⅲ randomised-controlled trials (RCT), such as RTOG 9704 and ESPAC 1/3 have supported its role as a predictive biomarker of adjuvant gemcitabine responsiveness, where patients with hENT1 positive tumour had significant survival benefit from adjuvant gemcitabine as compared to patients with low hENT1 tumours[65,66]. However, a recent Phase Ⅱ RCT stratified by hENT1 expression (LEAP: Low hENT1 and Adenocarcinoma of the Pancreas) comparing gemcitabine *vs* CO-101 (lipophilic gemcitabine) in metastatic PC failed to demonstrate this in metastatic disease[4]. Though the reasons for this are still unclear, the discrepancy may be due to the use of different hENT1 antibodies for immunohistochemistry, and/or perhaps the significance of hENT1 as a predictive biomarker is different in the metastatic as compared to the adjuvant setting. LEAP was the first purposely designed biomarker stratified trial in PC with prospec-

tive tissue acquisition, further analysis of the available tissue samples may offer more insight into gemcitabine responsiveness biomarkers.

***nab*-Paclitaxel (Abraxane®):** Secreted Protein Acid and Rich in Cysteine (SPARC, also known as osteonectin) regulates extracellular matrix modeling and deposition and may act as a tumour suppressor or an oncogenic driver depending on its differential expression in epithelial and stromal compartments in different cancer types[67]. High stromal and low epithelial expression of SPARC has been shown to be a poor prognostic biomarker in PC[68,69] and based on its hypothesised function as an albumin "sticker", it was developed as a therapeutic target for *nab*-paclitaxel to enable "stromal depletion" and in turn, to improve drug delivery. A positive phase Ⅰ/Ⅱ study of gemcitabine plus *nab*-paclitaxel demonstrated in a biological sub-study that SPARC expression in the stroma, but not in the epithelium, co-segregated with improved survival in PC, and hence a candidate predictive biomarker for *nab*-paclitaxel responsiveness[70]. This led to the recently reported Phase Ⅲ MPACT (Metastatic Pancreatic Adenocarcinoma Clinical Trial) RCT comparing gemcitabine *vs* gemcitabine plus nab-paclitaxel which demonstrated the significant addition survival benefit of nab-paclitaxel in patients with metastatic PC[71]. However, data concerning SPARC as a predictive biomarker of nab-paclitaxel responsiveness are not currently available. Although the relationship between SPARC expression and *nab*-paclitaxel responsiveness is still evolving, these proof-of-concept data suggest it warrants further exploration.

Improving treatment delivery: Targeting stroma

There is mounting evidence that stromal factors may be crucially important not only in determining the development and behaviour of carcinoma, but in influencing treatment response and, ultimately, prognosis. Stromal and epithelial cells may interact through direct cell-cell contact, or *via* paracrine signaling, and various non-cellular components in the stroma may influence either or both cell types. Many of these factors may contribute to cancer progression and metastasis through altered cell adhesion, epithelial-mesenchymal transition (EMT), matrix remodeling (facilitating tumour cell migration), and neovascularisation. These concepts have been examined in more detail elsewhere[72].

Individual differences in gene expression have been demonstrated within the stromal component of breast tumours, and these different phenotypes correlated with clinical outcome[73,74]. Differential expression of some of these same genes at the protein level appears to correlate with tumour regression in irradiated rectal carcinoma (Hemmings, unpublished data). One such protein is SPARC (Secreted Protein Acidic and Rich in Cysteine), a matricellular protein which modulates cell-cell and cell-matrix interactions, as described previously. Treatment with SPARC can block fibroblast activation and may

serve to inhibit angiogenesis[75]. There is some evidence that SPARC may act as a chemosensitiser by potentiating apoptosis[76]. SPARC may be upregulated in pancreatic cancer, and suppressing its expression may inhibit cancer cell migration, offering a potential therapeutic target[77]. Modulation of other matricellular proteins has also been shown (at least in a murine model) to alter chemotherapy response, without directly altering drug delivery[78], and the addition of agents which modify the tumour stroma may enhance chemotherapy response in clinical cases of operable pancreatic cancer[79].

Another important stromal variable is the host immune response to invading tumour cells. Whilst generally thought to be part of the host's armamentarium against cancer, it has become clear that inflammatory cells may promote the formation and progression of some tumours, and the balance of pro- and anti-tumour effects varies between individuals as well as between different tumour types. In pancreatic cancer, tumour-infiltrating T_H17 (lymphoid) cells may act on stroma to induce angiogenesis, as well as activating other tumour-promoting transcription factors[80]. In one model, tumours which were resistant to VEGF inhibitors were rendered sensitive by inhibition of T_H17 effector function, suggesting that immunomodulation may improve the efficacy of antiangiogenic treatments[81]. Similarly, tumour-associated macrophages (TAMs) may produce various growth factors as well as proteases which degrade the extracellular matrix, facilitating tumour invasion and angiogenesis[73], and may promote EMT in pancreatic cancer cells[82]. Transition of normal macrophages to tumour-promoting TAMs may be induced by IL-4 produced by pancreatic carcinoma cells[83], again offering a possible therapeutic target, and other inflammatory mediators may serve as biomarkers of prognosis in patients with advanced pancreatic carcinoma[84].

DEVELOPING THE EVIDENCE BASE FOR A ''PERSONALISED APPROACH''

Many challenges exist in developing the evidence base for a "personalised approach" to PC treatment. These include: appropriate design of clinical trials; development, interpretation and accreditation of standardised tests; matching appropriate patients to suitable trials; and minimising turn around time of new molecular based diagnostic tests required for trial eligibility and ultimately treatment selection[26,85,86]. A number of clinical trials examining different aspects of personalised treatment for pancreatic cancer are ongoing (Table 2). Biobanking of tissue samples linked to clinical outcomes data is possible within clinical trials and community cohorts. Such resources hold significant potential for true translational research.

Molecular profiling of tumours and the role of biobanks

Next generation sequencing is providing unprecedented opportunities to uncover the underlying genetic pathways driving cancer and is accelerating the drive towards personalized medicine. Human specimens that are analyzed using these technology platforms are a critical resource for basic and translational research in cancer because they are a direct source of molecular data from which targets for therapy, detection, and prevention are identified. The recent Federal Drug Administration approval of next generation sequencing platforms for diagnostic use (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm375742.htm) and the rapidly falling costs of whole genome sequencing will bring this technology into the clinic in the near future.

Biobanking has the potential to be a powerful platform for health innovation and knowledge generation, as biospecimens represent essential materials that fuel the advance of technology, scientific and medical research. This has stimulated a growing demand for appropriately qualified, well annotated biospecimens world-wide.

However, establishing a biobank of value, presents unique ethical, logistical, scientific, informational, and financial challenges in tissue acquisition and resource development. To be of maximum value tissue samples and analytical methods must be "fit for purpose" and reproducible.

Controlling pre-analytical variables is critical to ensure that the results of multidimensional high-throughput profiling are accurate and reproducible. The Australian Pancreatic Cancer Genome Initiative, an Australian led, international effort to characterise the genome of pancreatic cancer, has led international efforts to harmonise and standardize biospecimen collection, processing and downstream application. Factors critical to the success of this initiative include using best practice to guide processes, collection of multiple aliquots of specimens, ensuring all samples have a reference germline sample and expanding the repertoire to include the development of patient derived xenografts and cell lines. It is crucial to set appropriate standards from the projects initiation, and the human aspects of this complex enterprise cannot be underestimated to ensure quality samples that accurately represent the spectrum of cancer.

Meeting the challenges of biospecimen quality and interoperability requires a more modern approach to biobanking. Modern biobanking sees a new type of biospecimen emerge: where biospecimens are collected at distinct time points, and in a pre-specified clinical context. These samples are comprehensively annotated with clinico-pathological and treatment data, and linked to genomic and molecular data sets. Procurement of these types of samples requires a new organisational structure that incudes specific clinical disciplines such as interventional radiology and molecular pathology.

CONCLUSION

Recent advances in the treatment of pancreatic cancer have evolved through greater understanding of clinical tumour biology. None of this would be possible without access to tumour tissues. Biospecimen collection for

future research is becoming an integral part of trials and increasingly part of practice. Appropriate methods for collection, analysis and annotation of specimens are critical for maximising benefit from this valuable resource and ensuring reliability and reproducibility of results.

There is still much progress to be made in improving outcomes for patients with pancreatic cancer. Oncologists are increasingly recognising the importance of biospecimen collection to facilitate precision medicine. To make this a reality in practice, engagement of patients and other related clinicians(gastroenterologists, radiologists and pathologists) is vital. Acceptability to patients in routine practice is a crucial step in moving not just from bench to bedside but from trial to clinic.

The contribution of patients in allowing their specimens to be accessed for research cannot be undervalued. At both global and individual levels, for contribution to research and for personalisation of treatment, tissue is -and will continue to be- an important issue.

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2014 ADVANCES IN PANCREATIC CANCER

Imaging diagnosis of pancreatic cancer: A state-of-the-art review

Eun Sun Lee, Jeong Min Lee

Eun Sun Lee, Jeong Min Lee, Department of Radiology, Seoul National University Hospital, Seoul 110-744, South Korea

Jeong Min Lee, Institute of Radiation Medicine, Seoul National University College of Medicine, Seoul 110-744, South Korea

Author contributions: Lee ES reviewed the literature and wrote the paper; Lee JM contributed to the conception and design of the article, and approved the final version for publication.

Correspondence to: **Jeong Min Lee, MD,** Institute of Radiation Medicine, Seoul National University College of Medicine, 101 Daehangno, Chongno-gu, Seoul 110-744, South Korea. jmsh@snu.ac.kr

Telephone: +82-2-20723154 Fax: +82-2-7437418

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Abstract

Pancreatic cancer (PC) remains one of the deadliest cancers worldwide, and has a poor, five-year survival rate of 5%. Although complete surgical resection is the only curative therapy for pancreatic cancer, less than 20% of newly-diagnosed patients undergo surgical resection with a curative intent. Due to the lack of early symptoms and the tendency of pancreatic adenocarcinoma to invade adjacent structures or to metastasize at an early stage, many patients with pancreatic cancer already have advanced disease at the time of their diagnosis and, therefore, there is a high mortality rate. To improve the patient survival rate, early detection of PC is critical. The diagnosis of PC relies on computed tomography (CT) and/or magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP), or biopsy or fine-needle aspiration using endoscopic ultrasound (EUS). Although multi-detector row computed tomography currently has a major role in the evaluation of PC, MRI with MRCP facilitates better detection of tumors at an early stage by allowing a comprehensive analysis of the morphological changes of the pancreas parenchyma and pancreatic duct. The diagnosis could be improved using positron emission tomography techniques in special conditions in which CT and EUS are not completely diagnostic. It is essential for clinicians to understand the advantages and disadvantages of the various pancreatic imaging modalities in order to be able to make optimal treatment and management decisions. Our study investigates the current role and innovative techniques of pancreatic imaging focused on the detection of pancreatic cancer.

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Key words: Pancreatic neoplasms; Multi-detector computed tomography; Magnetic resonance imaging; Ultrasonography; Endoscopic ultrasound-guided fine needle aspiration; Positron-emission tomography

Core tip: To improve the survival rate of pancreatic cancer, early detection and optimal treatment with various imaging modalities is essential. Our study investigates the current role of pancreatic imaging, including computed tomography (CT), magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography, and biopsy/fine-needle aspiration using endoscopic ultrasound, focused on the pancreatic cancer. This study introduces rapidly-developing novel imaging techniques, including dual energy, low-tube-voltage CT techniques, iterative reconstruction CT algorithms, functional MRI methods, and hybrid positron emission tomography/MR, which are expected to become widely used and to show excellent performance for pancreatic cancer imaging in the near future.

INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related mortality worldwide, with an incidence rate equaling that of its mortality rate[1-3]. Whereas there have been great advances in the early detection and treatment of other malignancies such as colorectal cancer, breast cancer, and prostate cancer, the prognosis of pancreatic cancer is still bleak, as the five-year survival rate is less than 5% and the mortality rate has not declined over the last few decades[4,5]. Therefore, pancreatic cancer seems to remain one of the greatest challenges in the fight against cancer in the 21st century[6]. One of the main causes of the poor prognosis of pancreatic cancer is the difficulty of its early diagnosis. As pancreatic cancer typically develops with few symptoms in the early stage and there are not many specific, well-known risk factors aside from smoking and family history, the appropriate screening and early diagnosis of pancreatic cancer is quite challenging[7]. Therefore, only 10% to 20% of diagnosed patients have a chance of successful resection and possible cure, and even in patients with resectable disease, the survival rate is only 23%[3].

Despite the numerous obstacles detailed above, there is a continued effort to achieve early detection and to make the appropriate selection of surgical candidates with pancreatic cancer[8-12]. Furthermore, currently available pancreatic imaging has a key role in the characterization of pancreatic focal lesions, initial staging, surgical and therapeutic planning, and assessment of the treatment response using various imaging modalities, including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), and endoscopic ultrasonography (EUS)[8-18]. Multi-detector row computed tomography (MDCT) has a major role in the diagnosis and staging of pancreatic malignancies. MDCT of the pancreas is favorably complemented by EUS, which is more sensitive for the early detection of lesions, and allows relatively easy access to the pancreas for tissue diagnosis using fine-needle aspiration (FNA), as well as providing further important information for use in tumor staging[19].

MRI with magnetic resonance cholangiopancreatography (MRCP) and PET scanning can also have a successful role as a secondary imaging modality under special circumstances when CT and EUS are not diagnostic. Our study provides an overview of the current role and innovative techniques of pancreatic imaging for the detection and treatment of pancreatic cancer.

ROLE OF PANCREATIC IMAGING

Although the average survival time of patients resected for PC is approximately 12 to 20 mo, and there is a high probability of relapse due to the highly adverse and aggressive nature of the evolving disease, the primary treatment offering the greatest potential for cure is the complete, curative, surgical resectioning of the primary carcinoma[20,21]. As surgical and oncological treatments for pancreatic cancer have continually become more aggressive and sophisticated, the role of imaging has become more important, not only for initial diagnosis and staging, but also for determining both the resectability and the optimal treatment monitoring of pancreatic cancer[16,17,22]. MDCT is currently the worldwide imaging modality of choice for evaluation of pancreatic cancer, although ultrasonography, endoscopic US, contrast-enhanced US, and MRI with MRCP provide complementary, sometimes even more detailed, information[10]. Each imaging modality has both its advantages and disadvantages according to the four, different aspects regarding pancreatic cancer imaging evaluation: (1) identification of the primary tumor; (2) local tumor resectability; (3) distant metastasis; and (4) treatment monitoring.

US

US is frequently the first-line diagnostic tool for patients presenting with jaundice or abdominal pain, as it is a non-invasive and cost-effective modality. A hypoechoic mass, dilatation of the pancreatic duct, and dilatation of the bile duct are typical imaging features of pancreatic head tumor when seen on US. However, in cases of pancreatic body and tail cancers, tumor detection is quite difficult due to the lack of biliary dilatation and the presence of gas bubbles in the stomach and transverse colon, which cause posterior shadowing. In this situation, oral administration of water or other contrast agents may help to delineate the entire organ. The sensitivity and accuracy of pancreatic US is also highly dependent on the operator's experience, the degree of disease progression, and the body habitus of patients. For these reasons, the US sensitivity for detecting pancreatic cancer is controversial and has been reported as anywhere between 50%-90%[9,15,23-25]. Using US without contrast media, it is difficult to differentiate pancreatic cancer from other focal lesions, such as neuroendocrine tumor or chronic pancreatitis, as they show the same imaging features on conventional US. Overall, transabdominal US is an acceptable first-imaging method, although not a reliable method for a confident diagnosis or the exclusion of small pancreatic tumors, which are the only ones with even a slight chance for a cure[26].

CT

In many medical institutions, MDCT is routinely used as the most important pre-operative examination in patients with suspected pancreatic cancer, as it has good spatial and temporal resolution with wide anatomic coverage, and thus permits both comprehensive local and distant disease assessment during a single session[10]. In particular, among the cross-sectional imaging modalities, MDCT has shown the best performance for the evaluation of vascular involvement, which is the most important factor for predicting the tumor resectability[27-33]. The reported positive predictive value, sensitivity, and specificity for predicting the resectability of pancreatic cancer were 89%, 100%, and 72%, respectively[34]. In

terms of treatment monitoring following chemotherapy or surgery, MDCT is the primary imaging modality, and is used in conjunction with PET/CT[14,18]. However, MDCT may not depict small metastases to the liver or peritoneum[30], or even a primary pancreatic tumor showing isoattenuation[35].

EUS-FNA

As EUS offers excellent visualization of the pancreas from the duodenum or stomach and can produce high-resolution images of the pancreas, it has been considered one of the most accurate methods for the detection of pancreatic focal lesions, especially in patients with small tumors of 3 cm or less[36,37]. EUS also has the unique ability to obtain specimens for histopathological diagnosis using EUS-guided FNA. Since its early introduction in the early 1990s, EUS-FNA has emerged as a safe and accurate imaging technique for tissue diagnosis in patients with pancreaticobiliary disorders, particularly those with diagnosed pancreatic cancer. Furthermore, EUS-FNA has replaced endoscopic retrograde cholangiopancreatography (ERCP) with brush cytology as the endoscopic test of choice for tissue acquisition due to its higher success rates and decreased risk of post-procedural complications, especially in patients without obstructive jaundice[38]. Although EUS alone has shown slightly disappointing accuracy for differentiating pancreatic cancer from chronic pancreatitis (*i.e.*, 76% for malignancy and 46% for focal inflammation[37]), the reported sensitivity and accuracy of conjoined EUS-FNA for detecting pancreatic malignancy usually exceeds 90%[39-44]. According to a recent meta-analysis covering the years between 1995 and 2008, the pooled sensitivity and specificity of EUS-FNA were 86.8% and 95.8%, respectively, for diagnosing a solid pancreatic mass[38].

In order to improve diagnostic accuracy of EUS, contrast-enhanced EUS and EUS elastography are valuable new techniques to be considered. By administrating micro-bubble agents, the diagnostic accuracy of EUS can be as high as 82% for pancreatic adenocarcinoma[45]. EUS elastography, one of the most recent advances in gastrointestinal endoscopy, is a non-invasive technique that measures tissue elasticity in real time using a dedicated probe and system. A number of recent investigations have shown promising results of EUS elastography for diagnosing pancreatic focal lesions[46-49].

MRI

Over the past few years, MRI scanners and imaging techniques have become more sophisticated, resulting in improvements in both imaging quality and diagnostic accuracy. Therefore, MRI with MRCP is currently used as a problem-solving tool for patients with pancreatic disease[50]. Given the greater soft-tissue contrast of MRI compared with that of CT, there are several specific situations in which MRI is superior to CT: small tumors, hypertrophied pancreatic head, isoattenuating pancreatic cancer, and focal fatty infiltration of the parenchyma[17]. Therefore, MRI has been proven to be outstanding for

characterizing pancreatic masses. MRCP is also a very successful and classical MR technique for non-invasively delineating the pancreatic ductal system, as well as a valuable alternative to ERCP[51]. MRCP is also very useful for detecting subtle ductal narrowing that may suggest the presence of a small mass. Moreover, MRCP is very useful for delineating the presence of stones as an alternative cause of biliary or pancreatic ductal dilatation[17,52,53]. Although MDCT currently has a major role in the evaluation of PC, MRI with MRCP allows more successful tumor detection at an early stage by allowing a comprehensive analysis of the morphological changes of the pancreas parenchyma, as well as that of the pancreatic duct[20].

PET

PET/CT is an established molecular imaging modality, with fluorine 18-fluorodeoxyglucose (FDG), a glucose analogue, being the most widely used radiotracer[14,54]. The reported sensitivity and specificity of FDG-PET for the depiction of pancreatic cancer are 46%-71% and 63%-100%, respectively[55]. However, FDG-PET is more sensitive for treatment monitoring following chemo-radiotherapy and for depicting tumor recurrence after resection than MDCT[22,56-59]. Its wide anatomic coverage, which allows the depiction of all possible evidence of metastases in the entire body, is one of the advantages of PET/CT[18], while its inherently low spatial resolution and false-positive results caused by normal physiologic FDG uptake are its well-known limitations[60,61].

STANDARD PROTOCOL FOR PANCREATIC CANCER EVALUATION

In our institution, EUS and PET/CT are not performed by radiologists. Therefore, we do not deal with the technical protocols of EUS and PET/CT in this section.

US

US examination of the pancreas is performed following a minimum fast of 6 h. The purposes of the fast are to improve visualization of the pancreas, limit bowel gas, and ensure an empty stomach. US scan plans include transvers, longitudinal, and oblique scans along the pancreatic duct. Bowel gas can be displaced by moving the transducer and applying compression when necessary. To obtain complete visualization of all the portions of the pancreatic gland it is possible, and sometimes convenient, to employ different scanning techniques, such as filling the stomach with water, examining the patient with suspended inspiration or expiration, and changing the patient's position to erect, supine, and left and right decubitus. If the pancreas is poorly visualized, the water technique, using 100 to 300 mL of water through a straw, may be helpful[62].

CT

A pancreas-specific protocol for pancreatic cancer typi-

Table 1 Minimum technical specifications for pancreas computed tomography

Feature	Specification	Comment
Scanner type	Multi-detector row scanner	
Detector type	Minimum of four detector rows	
Reconstructed slice thickness	Minimum of 5 mm	Thinner slices are preferable, especially in multiplanar reconstructions (MPR)
Injector	Power injector, preferably dual-chamber	Bolus tracking desirable
Contrast injection rate	No less than 3 mL/s of contrast, 300 mg I/mL or a higher concentration, For a dose of 1.5 mL/kg of body weight	A saline flush desirable
Mandatory dynamic phases	1. Early arterial phase 2. Pancreatic phase 3. Portal venous phase	MPR, Curved MPR along the pancreatic duct, Minimum intensity projections are helpful

cally utilizes a thin-section, multi-phase technique with pre-contrast images and early arterial phase (CT angiography phase) images of the aorta and the superior mesenteric artery (17-25 s after the start of contrast injection), pancreatic phase (35-50 s after the start of contrast injection), and portal venous phase images (55-70 s after the start of contrast injection). Pancreatic phase images show peak pancreatic parenchymal enhancement, and therefore provide the best lesion to pancreas contrast. Portal phase images are helpful to assess the extent of venous involvement and to identify possible liver metastases[34,63-66]. The bolus tracking technique is currently routinely used to adjust for variations in the cardiac circulation time[34]. With regard to post-processing, a variety of techniques have been described for pancreatic imaging. The most commonly-used techniques are multiplanar reformations (MPR), curved multiplanar reformations (CMPR), and minimum intensity projections (MinIP)[65,67]. Oblique coronal or sagittal MPR and CMPR along the pancreatic duct can clearly demonstrate the relationship between tumors and the pancreatic duct or adjacent major structures. MinIP images use the lowest density values along each ray and clearly show low-density structures such as pancreatic and bile ducts. The recommended MinIP slab thickness is 3 mm for the pancreatic duct[65,66,68]. Maximum intensity projections are also often used to evaluate the relationship between tumors and adjacent, enhanced vessels.

In our medical institution at the time of our study, quadruple-phase CT images were obtained according to our biliary-pancreas protocol. First, a baseline, unenhanced scan was obtained from the hepatic dome to the third portion of the duodenum. After unenhanced scanning, patients received 1.5 mL/kg of iopromide (Ultravist 370; Schering, Berlin, Germany) intravenously for 30 s using a power injector at a rate of 3-5 mL/s. Triple-phase, dynamic CT scans were then obtained. The scanning delay for the arterial, pancreatic, and portal-venous phases was approximately 25 s, 40 s, and 70 s, respectively, after the initiation of the contrast injection. For MDCT scanners, a bolus-tracking method was used. After reaching an enhancement of up to 100 Hounsfield units in the descending aorta, as measured using the bolus-tracking technique, the scanning delay for the arterial phase was 5-6 s for all MDCT scanners. The scanning delay for the pan-

creatic phase was 19-22 s and that for the portal-venous phase was 52-65 s following contrast injection. The time required to reach 100 Hounsfield units in the descending aorta ranged from 18 to 23 s. For clinical interpretation, the CT images were reconstructed with a slice thickness of 2.5-3.0 mm and a reconstruction interval of 1.5-2 mm for MDCT[69]. The minimum technical specifications for MDCT of the pancreas are summarized in Table 1.

MRI
In many medical institutions, patients fast for four to six hours before MRI examination so that the gallbladder is distended and the signal from the overlying stomach and duodenum is decreased. For full evaluation of the pancreatic parenchyma and the pancreaticobiliary ductal system, obtaining the following MR sequences is recommended[50]: T1-weighted gradient-echo; T2-weighted axial and coronal sequences, usually turbo spin-echo; two dimensional (2D) and three dimensional (3D) MRCP; and T1-weighted 3D gradient-echo (GRE) before and after intravenous administration of gadolinium. Diffusion-weighted imaging (DWI) is currently becoming an increasingly used, optional sequence for the detection and characterization of pancreatic lesions[70]. The minimum technical specifications for MRI of the pancreas are summarized in Table 2. In our clinical practice at the time of our study, unenhanced T2-weighted images are usually obtained using a single-shot, fast SE sequence or a half-Fourier rapid acquisition with relaxation enhancement sequence. Unenhanced T1-weighted images are commonly acquired using in-phase and opposed-phase spoiled GRE (T1-weighted, dual-echo GRE) techniques. In addition, the following three MR cholangiographic methods were used to evaluate biliary anatomy: (1) the breath-hold, single-section, rapid acquisition with relaxation enhancement technique with fast or turbo SE sequences; (2) the breath-hold, multisection, single-shot, fast SE or half-Fourier rapid acquisition with relaxation enhancement technique; and (3) the respiratory-triggered, 3D, fast SE technique. Dynamic images were obtained using one of two fat-suppressed, 3D GRE sequences (*i.e.*, LAVA [liver acquisition with volume acceleration], GE Medical Systems and VIBE [volume interpolation with breath-hold examination], Siemens Medical Solutions) before and after the administration of gadolinium-based

Table 2 Minimum technical specifications for pancreas protocol magnetic resonance imaging

Feature	Specification	Comment
Scanner type	1.5-T or greater main magnetic field	Low-field magnets not suitable
Coil type	Phased-array, multichannel torso coil	Unless patient-related factors preclude the use
Gradient type	Current-generation, high-speed gradients (providing sufficient coverage of upper abdomen)	
Slice thickness	5 mm or less for dynamic series, 8 mm or less for other imaging	
Breath holding and matrix	Approximately 20 s of breath hold with a minimum matrix of 128 × 256	Breath hold instructions are very important
Injector	Power injector, preferably dual-chamber	Bolus tracking/MR fluoroscopy desirable
Contrast injection rate	1.5-2 mL/s of gadolinium chelate	Preferably resulting in the vendor-recommended total dose
Minimum sequences	T1-weighted, gradient echo (3D preferable) T2-weighted, turbo spin echo (axial, coronal) MRCP (both 2D and 3D preferable) Post-Gd, T1-weighted gradient echo	
Mandatory dynamic phases	Arterial Portal-venous phase Equilibrium phase	
Dynamic timing	Arterial: 20-40 s Portal venous: 45-65 s Equilibrium: 3-5 min after contrast injection	

MRCP: Magnetic resonance cholangiopancreatography.

contrast agents (Gd-BT-DO3A, Gadovist, Bayer Schering Pharma AG, Berlin, Germany) at a dose of 0.1 mmol per kilogram of body weight and with an injection rate of 1.5-2 mL/s (injection duration approximately 5-8 s). The arterial phase images were obtained five seconds after the gadolinium-containing bolus was detected in the abdominal aorta. Acquisition of 3D LAVA or VIBE data for each phase was completed during a single breath-hold at the end of expiration (mean time, 20 s; range, 18-21 s). Arterial, portal venous, and equilibrium phase images were obtained approximately 20-40 s, 45-65 s, and 3-5 min, respectively, after injection of the contrast agent. An additional, fat-suppressed LAVA or VIBE sequence was performed two minutes after the contrast-agent injection (between the portal venous and equilibrium phases) on the coronal plane and parallel to the portal vein bifurcation[71,72].

TYPICAL IMAGING FEATURES OF PANCREATIC CANCER

Pancreatic adenocarcinoma occurs most commonly in the pancreatic head (65%) and usually presents on US as a hypoechoic solid mass with ill-defined margins (Figure 1). Masses in the head of the pancreas cause ductal obstruction with secondary dilatation of both the common bile duct and the pancreatic duct, and result in the so-called double-duct sign[62]. On Doppler studies, pancreatic ductal adenocarcinoma shows poor vascularity[73], as well as poor enhancement on all phases of contrast-enhanced US[74]. This may be caused by marked desmoplasia, low mean vascular density, or the possible presence of necrosis and mucin[75].

On CT, pancreatic adenocarcinomas most often ap-

pear as hypoattenuating masses (Figure 2)[76]. However, approximately 10% of pancreatic adenocarcinomas are isoattenuating relative to the background pancreatic parenchyma[35], especially in small tumors 2 cm or less[77], thus making diagnosis more difficult. In these situations, indirect (secondary) signs, such as upstream pancreatic duct dilation or the double-duct sign caused by pancreatic and common bile duct obstruction, are helpful for diagnosis[76,77]. In addition, the pancreas distal to the tumor usually also appears atrophic. As the tumor grows, it typically infiltrates the peripancreatic structures and may result in encasement of adjacent vasculature and in some cases adjacent organs. Pancreatic cancers can occasionally appear to be cystic or necrotic, and in rare cases they can contain calcium[78].

On MRI, pancreatic cancer typically appears hypointense on fat-suppressed, T1-weighted imaging (Figure 3) and on pancreatic parenchymal phase, dynamically enhanced, fat-suppressed, T1-weighted sequences, whereas it has a variable appearance on T2-weighted images[79]. Pancreatic cancer also has a variable appearance on diffusion-weighted images. In a recent study of 80 patients, 38 pancreatic cancers appeared hyperintense, 12 isointense, and 4 hypointense[80].

Pancreatic adenocarcinoma usually manifests as an area of increased uptake on PET/CT and appears as a "hot spot" within the pancreas. On the basis of tumor biology and the degree of desmoplastic response, pancreatic ductal adenocarcinoma may demonstrate a low level of FDG uptake or none at all[14]. The reported SUV (standardized uptake value) of pancreatic adenocarcinoma (3.50 ± 1.66) was found to be higher than that of benign lesions (1.91 ± 0.65) and of the normal pancreas[81]. In a recent study of patients with suspected pancreatic cancer, the FDG uptake of malignant tumors
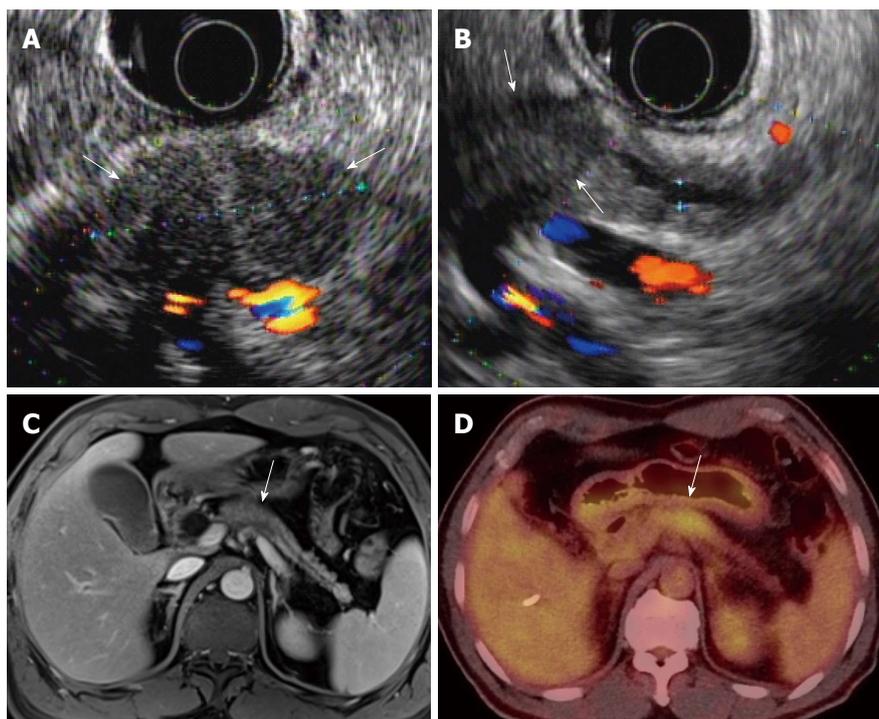
Figure 1 A 58-year-old, male patient with pancreatic body cancer with typical imaging findings. A, B: Endoscopic ultrasonography shows an approximately 3-cm, hypoechoic mass (arrows) in the pancreatic body with distal pancreatic duct dilatation; C, D: The mass (arrow) shows hypointensity on portal-venous-phase, contrast enhanced MR and hypermetabolism on PET/CT, respectively. MR: Magnetic resonance; PET: Positron emission tomography; CT: Computed tomography.

was also distinctly higher than that of benign lesions and in patients with chronic pancreatitis[55,82].

PERFORMANCE OF CT AND MR FOR DIAGNOSIS, STAGING, AND RESECTABILITY

With the continuing substantial improvements in CT technology, the capacity of MDCT for the detection, diagnosis, and local staging of pancreatic cancer has increased. MDCT is very effective for detecting and staging adenocarcinoma, with a sensitivity of up to 90% for detection and an accuracy of 80%-90% for staging[26]. Determination of the extent of vascular involvement is usually made by identifying the extent to which the tumor involves the cross-sectional circumference of a vessel. This can be done by identifying, with regard to the circular cross-section of a vessel, the degrees of circumferential involvement, as described by Lu *et al*[31]. Since their study was published in 1997, the terms "abutment" and "encasement" have also been used; abutment refers to the involvement of 180° or less of a vessel's circumference, and encasement refers to a greater than 180° vascular circumferential involvement[79]. As described above, as MDCT has shown the best performance for evaluating vascular involvement[27-33] (Figure 4), it is the most important factor for predicting tumor resectability. For example, four-section CT has been reported to have a 100% negative predictive value for vascular invasion and a 87% negative predictive value for overall tumor

resectability[30,83]. Therefore, MDCT is still the modality of choice for diagnosis and local staging of patients with pancreatic cancer.

Recently, distinct advances in MR technology have caused great improvements in pancreatic cancer imaging. At the same time, several literature reports have been published describing the comparable diagnostic performance of MDCT and MR[84-88]. According to a recent report by Koelblinger *et al*[85], the mean sensitivity and specificity of 64-detector row CT and 3.0-T MR imaging for the detection of pancreatic cancer (mean sensitivity, 95% *vs* 96%, respectively; mean specificity, 96% for both) do not differ significantly.

NEW TECHNIQUES IN PANCREATIC IMAGING

Dual-energy CT and low-tube-voltage techniques
Although MDCT has become the modality of choice for pancreatic cancer imaging and shows excellent performance regarding its diagnosis and staging, the detection of small pancreatic cancers < 2 cm in diameter or of isoattenuating tumors (which account for approximately 10% of all pancreatic adenocarcinomas) still remains challenging[35,77]. For those cases, we can improve the contrast-to-noise ratio between pancreatic cancer and normal parenchyma using the dual-energy or low-tube-voltage techniques[89]. A low-tube-voltage CT technique increases the X-ray absorption of iodine by increasing the gap between the mean effective energy of the X-ray
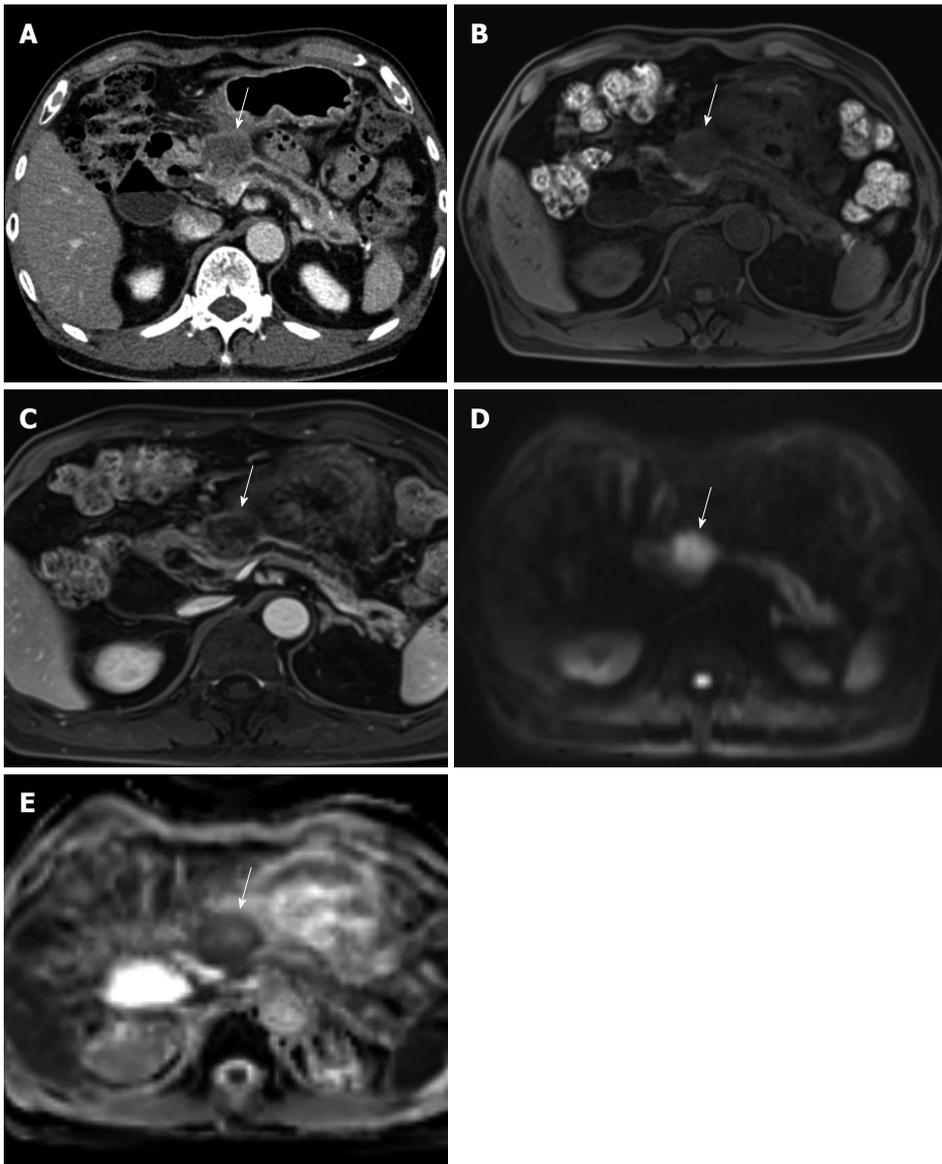
Figure 2 A 73-year-old male with pathologically-proven pancreatic head cancer. A: Approximately 3 cm low attenuating mass (arrow) is noted at the pancreatic head on the CT scan; B: In pre-contrast T1-weighted gradient echo sequence of MR, this mass (arrow) shows lower signal intensity, compared to the normal pancreatic parenchyma; C: After contrast media administration, the pancreatic head cancer (arrow) has poor enhancement; D, E: DWI with 1000 of b-value and ADC map reveal the diffusion restriction of the pancreatic head cancer (arrow). CT: Computed tomography; MR: Magnetic resonance; DWI: Diffusion weighted imaging; ADC: Apparent diffusion coefficient.

spectrum and the k edge of iodine[90]. Clinically, this phenomenon results in improved contrast enhancement of normal pancreatic parenchyma in order to maximize the contrast to typically poorly vascularized pancreatic cancers[90,91]. Therefore, dual-energy CT and low-tube-voltage techniques offer increased detection rates for small or otherwise isoattenuating pancreatic tumors[89-93].

Iterative reconstruction algorithm on MDCT
A new method for CT noise reduction based on iterative reconstruction (IR) algorithms has recently been developed. Since medical radiation exposure is generally increasing, one of the greatest concerns for radiologists, the use of this novel technique has recently been increasing due to its potential to preserve and enhance the diag-

nostic capability of CT with reduced radiation doses[94]. Currently, several IR methods have been proposed and are being commercially used for reducing radiation dose by decreasing the image noise during the reconstruction process (*i.e.*, adaptive statistical iterative reconstruction (ASiR, GE Health-care), model-based iterative reconstruction (MBIR, GE Healthcare), iterative reconstruction in image space (IRIS, Siemens Healthcare), sinogram-affirmed iterative reconstruction (SAFIRE, Siemens Healthcare), and iDose (Philips Healthcare)[95]. Based on its development, many studies have continuously revealed the superiority of IR, compared with routine filtered back projection, across the whole body[94-107]. Regarding the variety of reconstructing algorithms, each method may show a detailed difference in image quality,
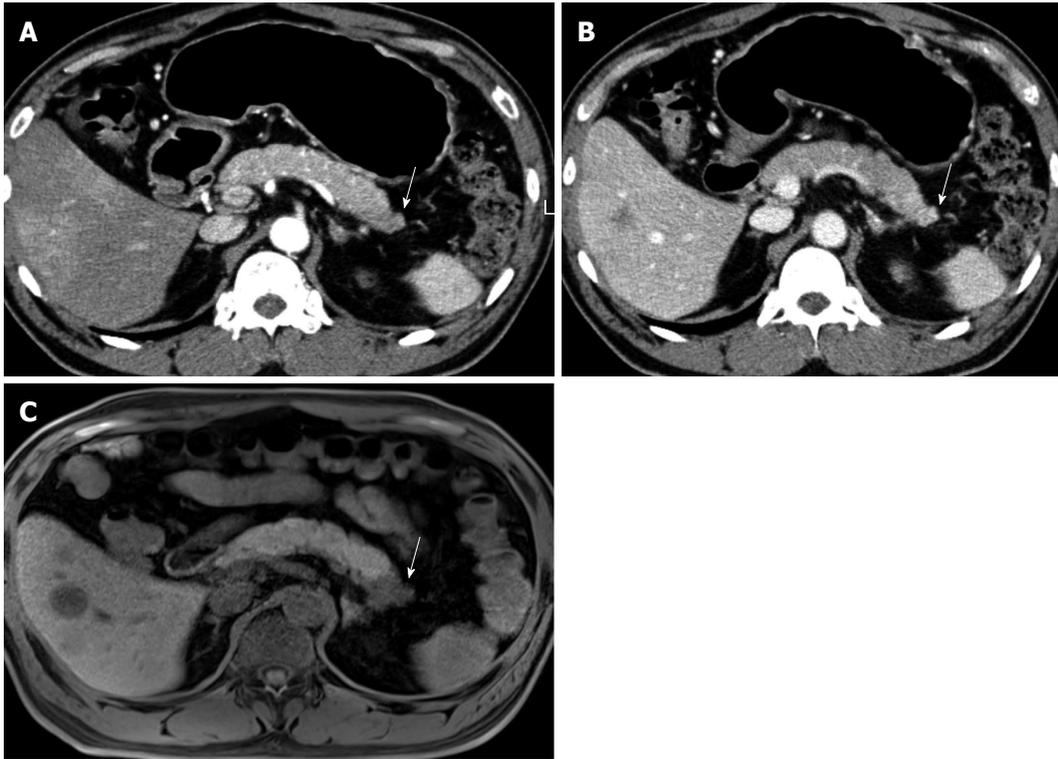
Figure 3 A 64-year-old male with biopsy-proven pancreatic adenocarcinoma with liver metastasis. A, B: On MDCT, the pancreatic tail mass (arrow) shows iso-attenuation, causing distal parenchyma atrophy; C: On pre-contrast, T1–weighted, gradient-echo sequence MRI, the pancreatic tail mass (arrow) is clearly depicted, as well as the liver metastasis, owing to the increased soft-tissue contrast of MR compared with that of CT. MRI: Magnetic resonance imaging; MDCT: Multi-detector computed tomography.
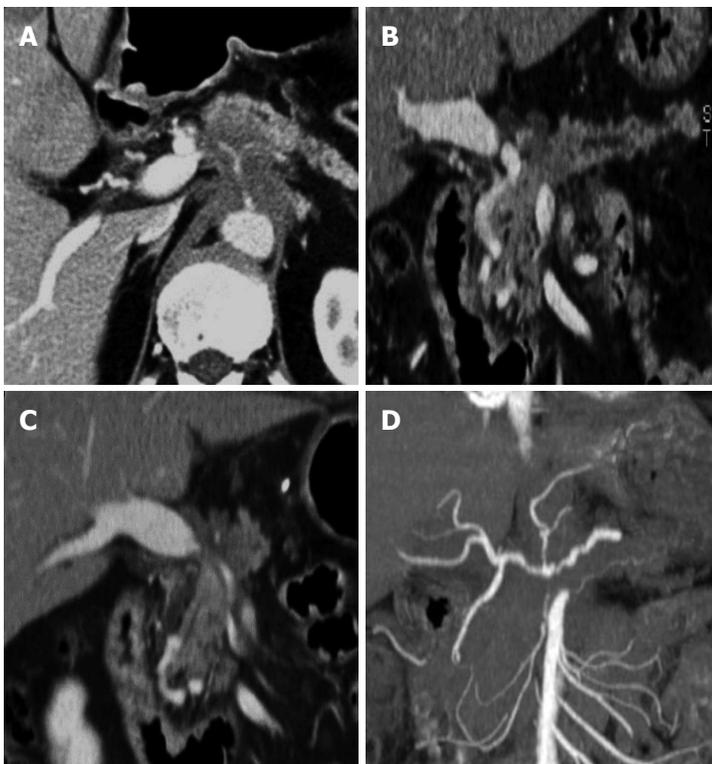
Figure 4 Post-process of multi-detector computed tomography for pancreatic cancer. A: In the axial CT scan, an ill-defined pancreatic body cancer invading the celiac axis is identified; B: On the curved MPR along the pancreatic duct, the relationship between the pancreatic duct and the cancer can be more easily understood; C, D: The extent and degree of major vascular involvement caused by the pancreatic cancer can be comprehensively assessed using MPR and the maximum intensity projections. MDCT: Multi-detector computed tomography; MPR: Multiplanar reformations.

as well as providing abnormal features such as a plastic, waxy, blotchy, or pixilated texture[104,107]. Considering the effects of IR techniques on reducing image noise, these techniques could be used for high spatial resolution pancreatic CT imaging, which may provide high quality, 1-2 mm, thin-slice CT images. Optimizing the IR technique
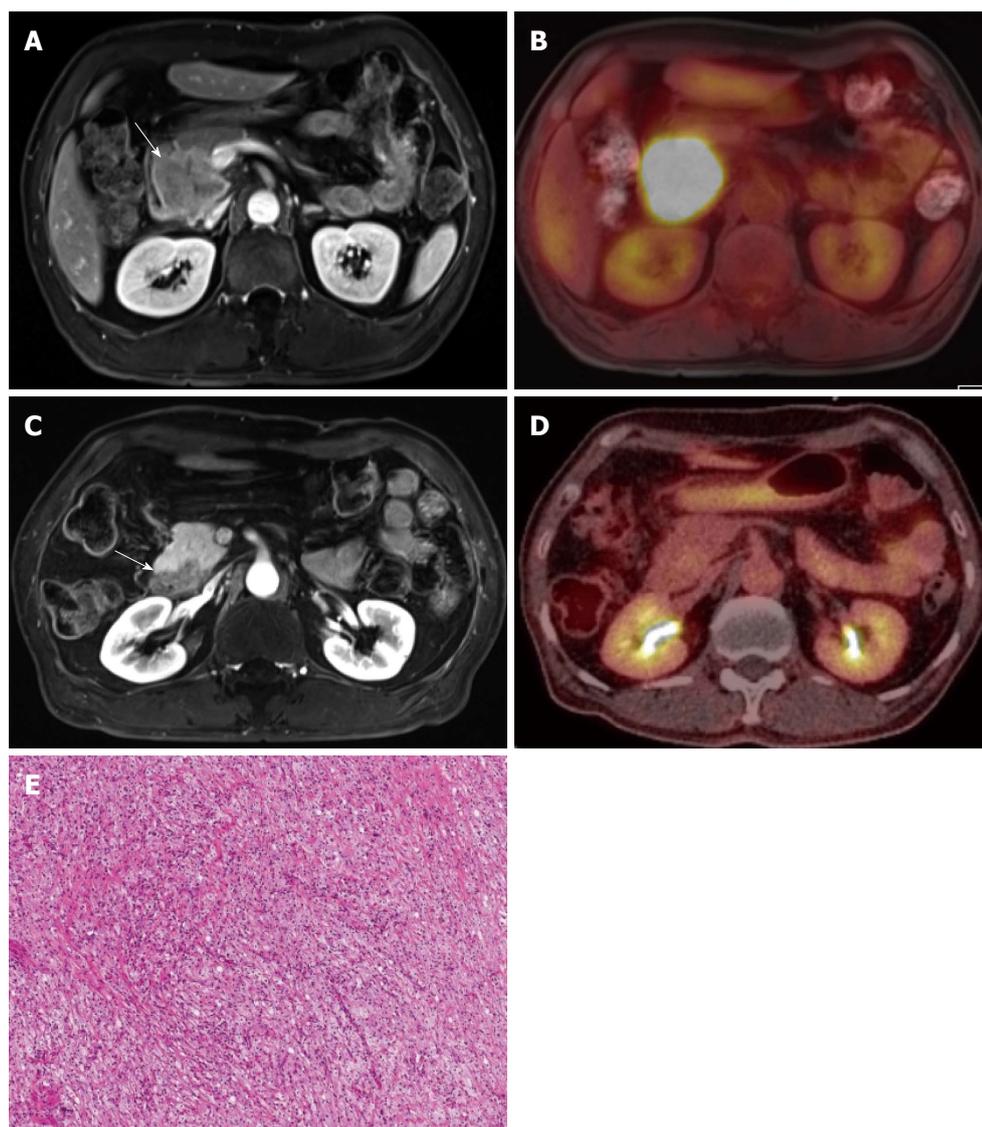
Figure 5 Treatment monitoring of pancreatic cancer using positron emission tomography/magnetic resonance. A, B: A 5-cm mass of biopsy-proven, adeno-squamous carcinoma (arrow) in the pancreatic head, as seen due to the strong FDG uptake; C, D: The mass (arrow) shows a marked decrease in size and glucose metabolism (from 22.0 to 3.8 of mSUV) after six cycles of neoadjuvant concurrent chemoradiation treatment. The specimen obtained during the following surgery revealed complete remission; E: All tumor cells are replaced by a foamy histiocytes collection of cholesterol clefts and multinucleated giant cells. PET: Positron emission tomography; MR: Magnetic resonance; FDG: Fluorodeoxyglucose; mSUV: Maximum standardized uptake value.

using a study protocol is necessary to balance imaging distortion, radiation reduction, and image quality, as well as high spatial resolution, along the z-axis.

Dynamic, contrast-enhanced-MR, DWI, and gadoxetic acid-enhanced liver MR for evaluation of liver metastasis
Although there is still technical complexity and room for improvement in terms of imaging resolutions regarding dynamic, contrast-enhanced (DCE) MR imaging, in previously published studies the quantitative analysis of the enhancement patterns and perfusion parameters using DCE-MR imaging has been shown to be both objective and helpful for the evaluation of malignant diseases regarding both their diagnosis and treatment monitoring[108-110]. In our preliminary data, the K(trans), K(ep), and iAUC values in patients with pancreatic cancer were

significantly lower than those seen in patients with a normal pancreas ($P < 0.05$), and were, therefore, useful for differentiating pancreatic cancer from pancreatic neuro-endocrine tumors[110].

DWI has also been used to characterize pancreatic lesions of various pathologic entities, such as cystic lesions, pancreatitis, and malignant tumors[70,111-113]. Although MR has the great advantage of excellent soft-tissue contrast for focal lesion detection, small or non-contour-deforming pancreatic adenocarcinomas may lack classic imaging features and thus may not be detected on conventional MRI. The use of diffusion-weighted imaging may allow earlier detection of pancreatic adenocarcinoma, as these neoplasms have increased signal intensity on diffusion-weighted images with high *b* values ($b > 500$ s/mm^2), as well as relatively low ADC values because of their

restricted diffusion associated with fibrosis[70]. The intra-voxel incoherent motion (IVIM) model takes these two sources of signal decay into account, thus providing a theoretical framework from which to derive diffusion and perfusion parameters from DWI[114]. Recently, the IVIM-approach with multiple b-values has been applied to pancreatic imaging, and there have been several reports showing promising results regarding the differentiation of pancreatic cancer from a normal pancreas[112,115]. DWI may also show small metastases that are not so clearly seen using other sequences and which, therefore, suggest to radiologists, on the basis of the high-signal-intensity lesion seen on diffusion-weighted imaging, to more closely examine the images obtained on other sequences[70,116]. Gadoxetic acid-enhanced liver MR imaging is also regarded as one of the best imaging tools for detecting liver metastasis in patients with pancreatic cancer. The reported sensitivity of gadoxetic acid-enhanced liver MR is 85% for detecting liver metastasis in pancreatic cancer, which is significantly higher compared with that of CT (69%)[117].

Hybrid PET/MR

Integrated PET and MR (PET/MR) scanners have recently become available for use in humans. As MR has the inherent strength of superior soft-tissue contrast resolution, multiplanar imaging acquisition, and functional imaging capability, such as that seen in DCE-MR, DWI, MR spectroscopy, or elastography, PET/MR may exhibit superior diagnostic performance compared with that of PET/CT[118]. In our medical institution, PET/MR imaging is now being used for evaluation of staging in patients with locally advanced pancreatic cancers, as well as for evaluation of tumor response in patients with pancreatic cancer undergoing neoadjuvant chemoradiotherapy before and after treatment (Figure 5).

CONCLUSION

There have recently been notable improvements in pancreatic imaging using the multi-modality approach, although each imaging modality has its own role, advantages, and disadvantages, not only for diagnosis, but also for the treatment and follow-up of pancreatic cancer. Both radiologists and clinicians should be familiar with those characteristics of imaging modalities, and apply them whenever possible. Rapidly developing, novel imaging techniques, including dual energy, low-tube-voltage CT techniques, IR algorithms, functional MR imaging methods, and hybrid PET/MR, are expected to become widely-used and to show excellent performance for pancreatic cancer imaging in the near future.

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c-Met signaling in the development of tumorigenesis and chemoresistance: Potential applications in pancreatic cancer

Daniel Delitto, Eva Vertes-George, Steven J Hughes, Kevin E Behrns, Jose G Trevino

Daniel Delitto, Steven J Hughes, Kevin E Behrns, Jose G Trevino, Department of Surgery, University of Florida-Gainesville, Gainesville, FL 32610, United States

Eva Vertes-George, Department of Pathology, University of Florida-Gainesville, Gainesville, FL 32610, United States

Author contributions: All the authors contributed to this manuscript.

Correspondence to: Jose G Trevino, MD, Department of Surgery, University of Florida-Gainesville, 1600 SW Archer Rd, Rm 6175, PO Box 100109, Gainesville, FL 32610, United States. jose.trevino@surgery.ufl.edu

Telephone: +1-352-2737967   Fax: +1-352-2650761

Abstract

Pancreatic ductal adenocarcinoma is the 4th leading cause of cancer deaths in the United States. The majority of patients are candidates only for palliative chemotherapy, which has proven largely ineffective in halting tumor progression. One proposed mechanism of chemoresistance involves signaling *via* the mesenchymal-epithelial transition factor protein (MET), a previously established pathway critical to cell proliferation and migration. Here, we review the literature to characterize the role of MET in the development of tumorigenesis, metastasis and chemoresistance, highlighting the potential of MET as a therapeutic target in pancreatic cancer. In this review, we characterize the role of c-Met in the development of tumorigenesis, metastasis and chemoresistance, highlighting the potential of c-Met as a therapeutic target in pancreatic cancer.

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Key words: Pancreatic adenocarcinoma; c-Met; Chemoresistance; Receptor tyrosine kinase

Core tip: As one of the leading causes of cancer-related deaths, pancreatic cancer remains elusive to our current therapeutic options. These modest advances in current therapies for pancreatic cancer have led to the recognition and development of targeted therapies toward tyrosine kinase receptors such as the c-Met receptor. In this review, we characterize the role of c-Met in the development of tumorigenesis, metastasis and chemoresistance, highlighting the potential of c-Met as a therapeutic target in pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the 4th leading cause of cancer deaths in the United States[1]. Currently, surgical resection is the only treatment option with the potential of cure. However, only 17% of patients are surgical candidates upon diagnosis and surgical resection in combination with chemotherapy and radiation therapy results in a 5-year survival of approximately 23% in specialized centers focused on pancreatic cancer[2]. While chemotherapy has the potential to delay tumor progression, innate or acquired chemoresistance and subsequent tumor resurgence is the norm[3,4]. Biologically diverse mechanisms have been identified to be involved in the chemoresistant phenotype, ranging from genetic and epigenetic changes to microenvironmental adaptation[4,5]. The goal of this review is to focus on the signaling of the mesenchymal-

epithelial transition factor protein (MET) in pancreatic cancer.

The mesenchymal-epithelial transition factor gene (*c-met*) encodes for a membrane-bound receptor tyrosine kinase (RTK) expressed predominantly by epithelial cells. MET is activated and signals downstream pathways following induction of phosphorylation in response to binding of its ligand, hepatocyte growth factor (HGF), also referred to as scatter factor. These ligands are secreted by cells of mesenchymal origin. The resulting HGF/MET pleiotropic signaling cascade activates mediators of cell proliferation and motility and has been heavily implicated in tumorigenesis *via* identification of amplification, activating mutation, and/or overexpression of MET in most solid organ neoplasms. Here, we review the literature to characterize the role of MET in the development of tumorigenesis, invasion, metastasis and chemoresistance, highlighting the potential of MET as a therapeutic target in pancreatic cancer.

PHYSIOLOGIC HGF-MET SIGNALING

MET activation propagates a complex system of intracellular signaling cascades that act to affect cell proliferation and migration. HGF is secreted by mesenchymal cells in close proximity to MET-expressing epithelial cells during embryogenesis or in response to tissue injury, thus functioning as a paracrine signaling mechanism that promotes cell proliferation and migration. MET is translated as a 180 kDa protein that is subsequently cleaved to form a heterodimer consisting of a short alpha (approximately 40 kDa) and long beta (approximately 140 kDa) chain of residues. The mature protein is then transported to and inserted in the plasma membrane. Upon HGF ligand binding to MET, autophosphorylation at multiple tyrosine residues within the cytoplasmic domain occurs, catalyzed by intrinsic ATPase activity. This results in changes in the tertiary structure of MET facilitating the formation of a signaling complex including GAB1 and GRB2 proteins that subsequently activates multiple downstream pathways (Figure 1). Known effector molecules of this signaling cascade include Src, mitogen-activated kinase, extracellular signal-regulated kinase 1 and 2, phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), signal transducer and activator of transcription (STAT), nuclear-factor-κB, and mammalian target of rapamycin[6-9]. MET-mediated induction of these pathways acts to positively influence cell proliferation, migration, and survival (Figure 2). *Via* these down-stream effectors, HGF-MET signaling plays a crucial role in important physiologic processes including embryonic development, organ regeneration and wound healing.

MET is essential for embryonic development and *hgf-* or *c-met*-null embryos die *in utero*[10]. In early embryonic development, HGF and its receptor MET are co-expressed by progenitor cells, suggesting autocrine signaling is an early homeostatic mechanism for stem cell survival[11]. HGF-MET signaling is necessary to ensure the growth and survival of placental trophoblast cells as well as embryonic hepatocytes. MET signaling is also necessary for the proper migration of muscle progenitor cells, development of the embryonic nervous system, and epithelial branching morphogenesis[12,13]. Later in development, paracrine HGF-MET signaling is critical for properly orchestrating organogenesis. Assays evaluating the ability of epithelial cells to form tubules *in vitro*, a process which recapitulates organ development, demonstrate that HGF signaling induces cells to undergo an epithelial-to-mesenchymal (EMT) transition. This transition allows host cells to relocate during embryonic development. Ultimately, these cells reclaim their epithelial identity, but the EMT marks a critical event in organogenesis.[11]

Inflammation and wound healing following injury are also highly dependent on HGF-MET signaling. HGF increases dramatically following renal or hepatic damage, inducing a diverse array of anti-apoptotic responses[9,14,15]. In cases of chronic or repetitive injury, HGF acts to oppose fibrosis by inducing apoptosis of myofibroblasts and by antagonizing transforming growth factor-β (TGF-β)[9,13,16]. Peptic ulcer disease represents a specific example of MET's protective effect. The loss of HGF signaling in a murine model led to decreased gastric mucosal cell proliferation and delayed healing from mucosal injury[17]. In fact, HGF-MET signaling has been implicated as essential to the protection, regeneration, and anti-fibrotic activity of cutaneous, pulmonary, hepatic, and gastrointestinal tissues in response to injury[13].

With respect to pancreatic endocrine physiology, the beta cell, responsible for insulin secretion, is dependent on HGF-MET signaling to hypertrophy and proliferate in response to persistent hyperglycemia[18]. In effect, MET is essential for the hyperinsulinemia seen in Type Ⅱ diabetes. *c-met* knockdown mice exhibit increased beta cell apoptosis during development and are more susceptible to streptozotocin-induced diabetes[19]. Additionally, *c-met* knockdown mice displayed reduced beta cell expansion during pregnancy leading to an increase in gestational diabetes[20]. Multiple investigations have confirmed that these knockdown mice have decreased glucose tolerance and reduced insulin secretion after stimulation[21,22]. In fact, stimulation of the HGF/MET pathway has been suggested to encourage beta cell proliferation after islet cell transplantation. Thus, MET plays a critical role in pancreatic neuroendocrine cell proliferation and development.

Relatively little data is available concerning MET signaling and normal pancreatic exocrine development. A recent investigation by Anderson *et al*[23] examined the phenotype of a point mutation in *c-met* that impaired localization and activation of MET. Zebrafish with this mutation exhibited mislocalization of pancreatic ductal cells compared with wild-type animals. Interestingly, ductal proliferation was unaffected. Further, inhibition of MET proteindownstream signaling with PI3K and STAT inhibitors produced a similar phenotype, suggesting an essential role for MET in migration and localization of
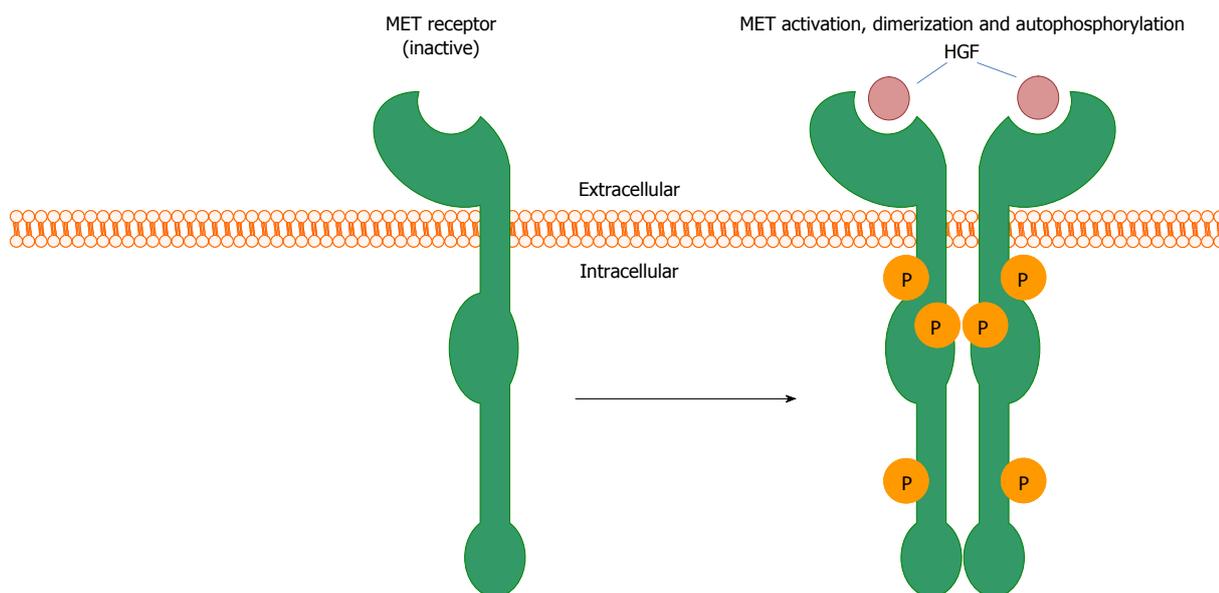
Figure 1 The mesenchymal-epithelial transition factor receptor functions as a transmembrane tyrosine kinase receptor. Ligand binding from hepatocyte growth factor (HGF)/scatter factor induces receptor dimerization and autophosphorylation of intracellular tyrosine residues, which serves as a catalytic site for the SH2 domains of numerous cytosolic signaling proteins. MET: Mesenchymal-epithelial transition factor.
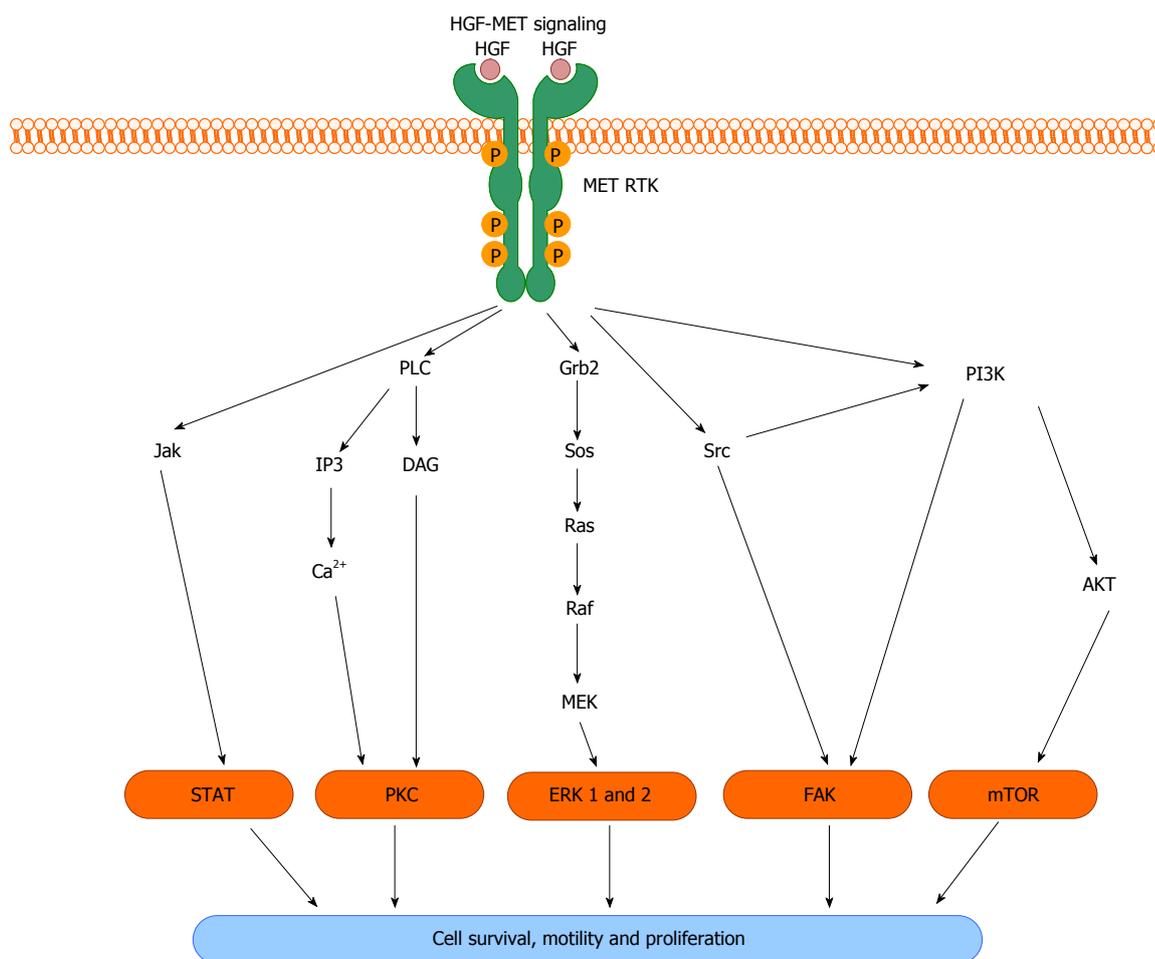
Figure 2 Hepatocyte growth factor activation of the mesenchymal-epithelial transition tyrosine kinase receptor induces a pleiotropic response involving a host of intracellular signaling to induce cell survival, migration and proliferation. HGF: Hepatocyte growth factor; MET: Mesenchymal-epithelial transition factor; RTK: Receptor tyrosine kinase; JAK: Janus kinase; STAT: Signal transducer and activator of transcription; PLC: Phospholipase C; IP3: Inositol triphosphate; DAG: Diacylglycerol; Ca^{2+}: Calcium; PKC: Protein kinase C; Grb2: Growth factor receptor-bound protein 2; Sos: Son of sevenless homolog; Ras: Harvey rat sarcoma viral oncogene homolog; Raf: Rapidly accelerating fibrosarcoma; MEK: Mitogen activated protein kinase kinase; ERK: Extracellular-signal-regulated kinase; FAK: Focal adhesion kinase; PI3K: Phosphoinositide 3-kinase; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin.

embryonic pancreatic ductal cells.

In summary, physiologic HGF-MET signaling is essential for appropriate embryonic development and organ repair. The function of the HGF/MET pathway observed in multiple organ systems appears to drive cell proliferation and mobility. Unfortunately, dysregulation of this pathway clearly could result in tumor initiation and/or progression. Amplification, mutation or over-expression of *c-met* become deleterious, contributing to malignant transformation and metastasis. Activating and sustaining HGF-MET signaling in this pathologic context drives tumor progression and is responsible, at least in part, to the development of chemoresistance.

PATHOLOGIC HGF-MET SIGNALING IN CANCER

Excessive MET activity is a feature of many cancers, although inciting mechanisms appear to be tumor-specific[24]. *c-met* received early attention as a proto-oncogene when activating mutant alleles were implicated in cases of hereditary papillary renal cell carcinoma[25]. The resulting MET receptor was constitutively activated, undergoing spontaneous ligand-independent phosphorylation[11]. In an analysis of seven families with hereditary papillary renal carcinoma, four displayed activating *c-met* mutations, all of which were located in the tyrosine kinase domain of the MET protein[25]. Sporadic *c-met* mutations have also been described in gastric carcinomas, glioblastomas, and squamous cell carcinomas of the head and neck[11,12,26]. Furthermore, aberrant positive feedback systems involving autocrine and paracrine signaling in the HGF-MET axis contribute to tumorigenic phenotypes in melanomas, osteosarcomas, breast cancer and gliomas[26]. One retrospective histopathologic analysis observed MET over-expression in 87% of renal cell carcinoma specimens[27]. Additionally, a strong correlation between MET expression and the esophageal metaplasia-dysplasia-adenocarcinoma continuum has been shown in surgical specimens from patients with esophageal adenocarcinoma[28]. In fact, *c-met* amplification occurs in approximately 9% of esophageal cancers[29]. These investigations provide compelling evidence that c-Met is a potent oncogene.

The association between MET activity and neoplastic progression has been investigated in animal models. Hypoxia-induced tumor cell invasion is dependent upon upregulated MET signaling, suggesting another mechanism driving growth and metastasis[30,31]. Overexpression of wild-type MET in hepatocytes led to spontaneous hepatocellular carcinoma development that regressed upon MET inactivation[30,32]. Thus, overexpression of non-mutated MET is sufficient to induce tumor development. Moreover, inhibition of MET caused established tumors to regress, suggesting that MET signaling is necessary for tumor growth and maintenance. Subsequent animal models have proposed that the frequency of many carcinomas and lymphomas is greatly increased by MET overexpression[53]. Non-neoplastic cell lines forced to constitutively express HGF or MET become highly tumorigenic when implanted *in vivo*[34,35]. Therefore, while MET activity may not be the inciting mechanism in the formation of many cancers, overexpression in pre-clinical models appears to confer a more aggressive phenotype.

In fact, MET expression has been correlated with more aggressive disease and worse clinical outcomes in many cancers. In NSCLC, MET overexpression correlates with an unfavorable prognosis and has been implicated as a primary mechanism of resistance to epidermal growth factor receptor (EGFR) inhibitor therapy[36,37]. In hepatocellular carcinoma the expression level of MET is directly correlated to metastatic behavior and inversely correlated to the level of tumor differentiation and patient survival[38-41]. In a prospective cohort analysis of 554 patients with renal cell carcinoma, a particular single nucleotide polymorphism (SNP) in *c-met* was associated with a decline in median recurrence-free survival from 50 to 19 mo[42]. While the functional outcome of this SNP remains to be elucidated, an activating point mutation is highly suspected. Likewise, MET overexpression is a HER2/neu-independent prognostic marker for node-positive breast cancer, signifying increased tumor aggressiveness[43]. MET expression significantly correlated with the depth of invasion and regional lymph node metastasis in colorectal cancer[44]. Thus, the list of solid organ neoplasms for which upregulation of HGF-MET signaling portends a more aggressive phenotype is extensive[45,46]. Taken together, this data demonstrates that dysregulation of the HGF-MET pathway contributes to tumor progression. This data also has implications regarding the status of the HGF-MET pathway on the effectiveness of certain biologic therapies, a concept we will expand upon later.

Concerning pancreatic adenocarcinoma, evidence is accumulating that correlates dysregulated MET activity with an aggressive phenotype. In a recent investigation thirty-six pancreatic tumor samples were analyzed and MET expression levels were directly proportional to tumor grade[47]. Similar histopathologic analyses showed an approximate five to seven-fold increase in MET protein expression levels in pancreatic cancer compared to normal pancreas samples[48,49]. Histopathologic evaluation of our own resected patient population support these findings (Figure 3). A larger collection of pancreatic tumor specimens subsequently confirmed increased MET protein expression compared with normal controls and MET protein overexpression significantly correlated with increased TNM stage[50]. In fact, secreted HGF protein from surrounding stromal tissue has been correlated with MET overexpression in patients with pancreatic cancer and associated with worsened overall survival[51]. Given the known pathophysiologic actions of MET in cancer and a well-demonstrated overexpression pattern in pancreatic adenocarcinoma, inhibition would seem a logical therapeutic avenue.

Unfortunately, targeting MET alone as a therapeutic strategy appears to be overly optimistic. Despite con-
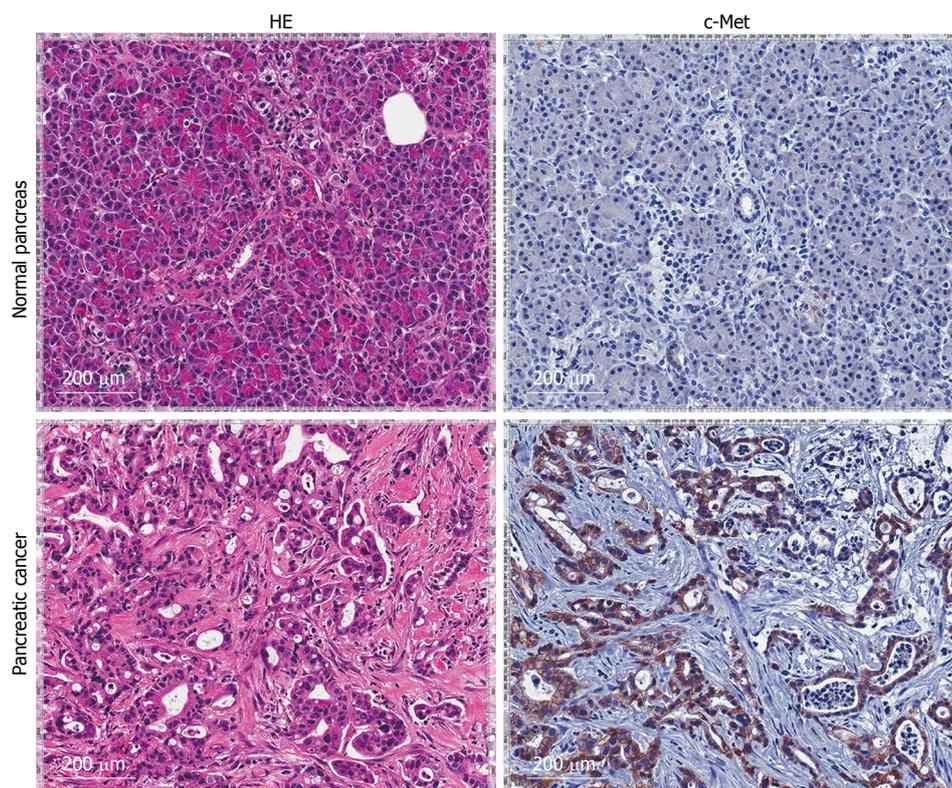
Figure 3 Immunoperoxidase staining. Immunoperoxidase staining of formalin fixed, paraffin embedded human pancreatic specimens demonstrate over expression of c-Met receptor in pancreatic cancer patients when compared to adjacent normal pancreatic tissue controls (right panel). HE staining demonstrate histological confirmation of diseased (pancreatic cancer) or normal tissue (left panel).

vincing evidence of primarily MET-induced tumors, a growing body of evidence supports secondary MET involvement in a synergistic crosstalk with other RTKs such as EGFR, vascular endothelial growth factor receptor and insulin-like growth factor-1 receptor (IGF-1R) to promote malignant cell migration, invasion, and chemoresistance[52-55]. In hepatocellular carcinoma cells, EGFR co-immunoprecipitates with MET and activated EGFR leads to ligand-independent activation of the MET pathway[36]. MET and IGF-1R synergistically promote migration and invasion in pancreatic adenocarcinoma. Downregulation of MET *via* adenoviral infection with a MET ribozyme abrogated the effects of IGF-1, suggesting codependence of IGF-1R and MET in directing tumor invasion and migration[56]. These complex, multifactorial interactions among RTKs play a key role in growth and maintenance of a variety of tumor types and are under intense scrutiny for potential therapeutic value or mechanisms of therapeutic resistance. These discoveries will be essential to the evolving reality of personalized cancer treatment strategies.

MET AND TUMOR METASTASIS

The microenvironment of a tumor may be as instrumental to the progression of disease as the tumor itself. In fact, stromal support in the form of angiogenesis, mitogenic signaling and cytoskeletal attachments are necessary for tumors to grow and metastasize *in vivo*. As previously

mentioned, HGF secretion by stromal cells mediates MET activity in a paracrine manner. Additionally, HGF-MET signaling encourages angiogenesis by inducing VEGF expression by cancer cells[57,58]. However, neovascularization alone is not sufficient for metastasis to occur.

Recall that in embryonic development and tissue repair, MET plays an essential, physiologic role in cellular migration and subsequent organogenesis. Unfortunately, overexpression of MET and its subsequent downstream pathways, including PI3K and Src, similarly enable growth and invasion of malignant cell populations. An initial step in tumor migration involves clearing a path through the extracellular matrix (ECM). This is accomplished primarily by the actions of secreted matrix metalloproteinases (MMPs), which digest surrounding ECM. Not surprisingly, MMPs have been shown to be upregulated by MET signaling[24].

Cells must also respond to chemotactic factors in the ECM for effective migration. As previously mentioned, an EMT endows epithelial cells with certain properties of mesenchymal cells that enable migration. Furthermore, it has recently been proposed that the EMT may be coupled with a transition to a more stem-cell-like state, suggesting further importance of the EMT to metastasis and tumor progression[59]. In embryogenesis, MET controls the EMT necessary to enable myogenic progenitor cell migration[9]. Additionally, EMT is further driven by Wnt signaling, a pathway that is also stimulated by MET *via* glycogen synthase kinase 3-β[60]. The mechanism by

Table 1 Cancer stem cell markers are listed with previously described functions

CSC marker	Proposed function
CD44	ECM binding, organization of actin cytoskeleton, modulation of mitogenic signaling[112]
CD24	P-Selectin binding, cell migration[113]
ESA	Mediation of epithelial intercellular adhesion[114]
CD133	Activation of Wnt signaling and angiogenesis[115,116]
CXCR4	Receptor of SDF-1, hematopoietic stem cell homing, invasion[117]
MET	Receptor of HGF, promotes cell growth, proliferation, migration[11]
u-PA	ECM degradation, cell migration[118]

Note the pattern of migratory functions associated with cancer stem cell (CSC) markers. ECM: Extracellular matrix; ESA: Epithelial specific antigen; CXCR4: Chemokine receptor type 4; SDF-1: Stromal cell-derived factor 1; MET: Mesenchymal-epithelial transition factor; HGF: Hepatocyte growth factor; u-PA: Urokinase-type plasminogen activator.

which MET governs the EMT directly in tumor metastases remains to be elucidated.

Finally, malignant cells must take up residence in a distant organ as a metastatic focus. Remarkably, HGF-MET signaling plays a role both in cellular dissociation within the primary tumor and cellular re-association within the metastatic niche[24]. HGF triggers destabilization of adherens junctions within the primary tumor through FAK-mediated integrin signaling[61]. As tumor cells invade and metastasize, failure of proper interaction with foreign microenvironments leads to programmed cell death. HGF-MET signaling upregulates cytoskeleton adhesion receptors and enables tumor cells to effectively engage their new surroundings and elude apoptosis, thereby facilitating metastatic development[24]. Thus, in addition to fostering primary tumor growth, MET appears to act at multiple regulatory points in the development of metastatic disease.

MET AND CANCER STEM CELLS

A growing body of evidence suggests that a hierarchy exists in cancer cell populations, a notion initially discovered in hematopoietic malignancies. Cancer stem cells (CSCs) actually comprise a small minority of tumor cells but appear to be the only group capable of unlimited self-renewal and formation of xenografts. Interestingly, these cells appear to have a limited potential for further differentiation[62,63]. CSC populations have subsequently been identified in a variety of solid organ neoplasms including brain, breast, melanoma, pancreas, prostate and colon. While CSC identification is specific to each tumor type, common themes include cell surface markers such as CD24, CD44, CD133, epithelial surface antigen (ESA), chemokine receptor type 4, and urokinase plasminogen activator (Table 1)[64-72]. Importantly, in pancreatic cancer stem cell (PCSC) populations, MET overexpression conferred an equally tumorigenic phenotype to CD44$^+$/CD24$^+$/ESA$^+$ cells[73]. Restated, MET overexpression alone may sustain a pan-

creatic cancer stem cell phenotype.

Conversely, MET overexpression may prompt cancer cells to dedifferentiate into CSCs. MET activation in prostate cancer cells induces a stem-like phenotype and endows cells with more invasive potential[74]. In head and neck squamous cell carcinoma, cells overexpressing MET can recapitulate the heterogeneity of parental tumors *in vivo* and exhibit increased self-renewal, invasion, and metastasis[75]. In glioblastomas, overexpression of MET leads to a stem-like phenotype resistant to terminal differentiation signals[76]. Regardless of the origin of CSCs, MET overexpression is associated with a stem-cell-like phenotype in a wide range of cancers.

MET AND CHEMORESISTANCE

Chemoresistance is an important factor contributing to the high mortality rate of most cancers and is germane to treatment failure in pancreatic cancer. With few exceptions, tumor metastasis precludes surgical therapy and leaves chemotherapy as the only therapeutic option. In borderline cases, neoadjuvant chemotherapy protocols may offer opportunities for attempts at a surgical resection. After surgery, adjuvant chemotherapy protocols are beneficial in avoiding recurrence, especially in more aggressive tumor types. Unfortunately, the development of chemoresistance is a real oncologic dilemma. In the face of chemoresistant tumor populations, no effective treatments exist. Therefore, understanding the molecular regulators of chemoresistance has major implications in therapeutic intervention. Several lines of evidence converge to suggest that MET overexpression may confer a chemoresistant phenotype.

We have outlined the close relationship between MET and CSCs. In fact, CSCs have been shown to be largely responsible for chemoresistant phenotypes in glioblastomas, hematopoietic, pancreatic and colorectal cancers[77-83]. Mechanisms range from reducing drug delivery to repairing cytotoxic injury and ultimately result in tumor cell repopulation[77-83]. Furthermore, a higher proportion of cells bearing CSC markers has been associated with poor outcomes in glioblastomas, breast and pancreatic cancer[84-86]. Thus, investigative directions have become particularly focused on identifying factors that drive and sustain CSCs. Given the significance of HGF-MET signaling in PCSC populations, the role of MET in this process would seem to be particularly relevant in pancreatic cancer.

The activation of the HGF-MET axis has been directly implicated in acquiring and maintaining chemoresistance in several tumor cell populations (Table 2). HGF stimulation protects NSCLC cells from cisplatin toxicity, in part mediated by downregulation of apoptosis-inducing factor[87]. *c-met* amplification is associated with NSCLC resistance to the EGFR inhibitor Gefitinib *via* modulation of the PI3K pathway[88]. Multiple investigations have revealed that MET inhibition sensitizes ovarian carcinoma to carboplatin plus paclixatel, whereas MET over-

Table 2 Mechanisms of hepatocyte growth factor-mesenchymal-epithelial transition factor induced chemoresistance in different cancer types

Cancer type	Chemotherapy	Mechanism of HGF-MET signaling in chemoresistance
Multiple myeloma	Bortezomib	MET overexpression: Apoptotic resistance *via* PI3K-Akt activation[92]
Glioblastoma	Radiation, cisplatin, camptothecin, adriamycin, and taxol groups	Addition of HGF: Anti-apoptotic effects *via* PI3K-Akt dependent pathways[91]
Rhabdomyosarcoma	Vincristine/etoposide, radiation	Addition of HGF: Enhanced migration, MMP secretion, PI3K-Akt activation[119]
Non-small cell lung carcinoma	Cisplatin	Addition of HGF: Downregulation of apoptosis-inducing factor (AIF)[87]
Non-small cell lung carcinoma	Erlotinib	*c-met* amplification: Activation of EGFR, preservation of PI3K-Akt activation[88]
Gastric adenocarcinoma	Adriamycin	Addition of HGF: Anti-apoptotic effects *via* PI3K-Akt upregulation[93]
Pancreatic adenocarcinoma	Gemcitabine	MET overexpression: Anti-apoptotic effects *via* PI3K-Akt activation, induction of EMT-like changes[94,95]
Ovarian adenocarcinoma	Carboplatin/paclitaxel	MET overexpression: Apoptotic resistance *via* PI3K-Akt activation[89,90]

MET: Mesenchymal-epithelial transition factor; PI3K: Phosphoinositide 3-kinase; Akt: Protein kinase B; HGF: Hepatocyte growth factor; MMP: Matrix metalloproteinase; EGFR: Epidermal growth factor receptor; EMT: Epithelial-mesenchymal transition.

expression imparts chemoresistance[89,90]. Furthermore, stimulation of the HGF-MET pathway confers protection against chemotherapeutic agents by upregulation of PI3K/Akt signaling in multiple myeloma, glioblastoma and gastric adenocarcinoma[91-93]. Our group has found that pharmacologic MET inhibition using a small molecule inhibitor sensitizes esophageal adenocarcinoma cells to pyrimidine analog chemotherapy (unpublished data). Additionally, preclinical studies have demonstrated that overexpression of MET has also been associated with EMT-like changes in acquired-gemcitabine-resistant pancreatic cancer cells[94]. These findings are not surprising as pancreatic cancer is known for rapid acquisition of chemoresistant behavior and also MET overexpression. Additionally, MET inhibition in pancreatic adenocarcinoma leads to gemcitabine sensitization[95]. Although consisting largely of *in vitro* data, these investigations demonstrate a strong correlation between MET overexpression and chemoresistance in a variety of malignancies.

The mechanism by which MET overexpression confers chemoresistance in pancreatic cancer likely involves the mesenchymal support network. Tumors most heavily invested with stroma are often those most refractory to chemotherapy[4]. Stroma is the predominant source of HGF, suggesting MET activation is, at least in part, a result of paracrine signaling. In breast cancer, HGF-MET signaling augments tumor cell adhesion to ECM components by upregulating integrin synthesis and inducing conformational changes that activate integrins[24,96]. This integrin-mediated adhesion is actually a mechanism by which tumor cells can oppose the cytotoxic effect of chemotherapy[97]. Indeed, studies have shown that integrin expression, specifically α β, is upregulated in cases of relapsed leukemia. This finding suggests that increased integrin expression may contribute to generating minimal residual disease, defined as tumor cell persistence following therapy[4]. Further investigation is necessary to characterize the mechanism by which MET-driven integrin upregulation imparts chemoresistance and whether this principle is applicable to other tumor types. However, disruption of the HGF-MET axis may result in biochemical dissociation from the protective mesenchymal environment, thereby imparting sensitivity to cytotoxic therapies.

Data specific to the pancreatic cancer microenvironment regarding MET signaling is forthcoming. Animal models that utilize VEGF inhibitors to impart ischemia actually result in increased tumor growth and invasion but inhibition of MET abrogates this proliferative response to hypoxia[98]. As previously mentioned, PCSCs can be defined by comparatively high MET expression. Pharmacologic inhibition of MET in PCSC populations blocked self-renewal capacity, reduced the overall PCSC population and significantly slowed tumor growth *in vivo*[99]. Treatment with MetMAb, a monovalent antibody against MET, has shown decreased pancreatic tumor growth in orthotopic models *in vivo*[100]. Further, recent preclinical data suggest cabozantinib, a novel small molecule MET inhibitor, overcomes gemcitabine resistance. These studies will likely lead to phase 3 clinical trials using this inhibitor in pancreatic cancer patients[101].

Finally, the interplay between RTKs and the potential for redundancy deserves emphasis when discussing therapeutic intervention. MET and other RTKs are involved in a complex signaling network that may exist as a redundant system with controlled feedback. For example, MET induction has been associated with anti-EGFR therapy and resultant MET overexpression confers resistance to EGFR inhibitors in lung and colorectal cancer[88,102-104]. Thus, MET inhibition may potentiate therapeutic effects aimed against other RTKs, and vice versa. In fact, effective siRNA inhibition of c-Met transcripts in NSCLC confers sensitization to gefitinib, an inhibitor of EGFR[88]. Further, concomitant administration of EGFR and MET inhibitors eliminated NSCLC cells more effectively than either drug alone[55,105]. Similarly, MET inhibition led to increased sensitivity of her2-positive breast cancer cells to trastuzumab[106]. Not surprisingly, combination RTK inhibition is quickly becoming the standard in targeted oncologic chemotherapies involving MET inhibition.

CONCLUSION

In summary, *c-met* encodes a versatile RTK crucial to physiologic cell proliferation, organogenesis and wound healing. Its mechanism of action involves multiple anti-apoptotic, pro-mitogenic, and pro-motility downstream

Table 3 Mesenchymal-epithelial transition factor inhibitors are shown with specific targets and evidence of anti-tumor effect

Drug	Target(s)	Impact
Cabozantinib	MET	Induced apoptosis in gemcitabine-resistant pancreatic cancer cell lines, currently in phase I clinical trials[101]
Crizotinib	ALK, MET	Inhibited growth of gemcitabine resistant pancreatic cancer cell lines[95], FDA approved for ALK-expressing NSCLC and myofibroblastic sarcomas
Foretinib	MET, VEGFR	Inhibited tumor growth in lung metastasis animal model but failed to show benefit in multiple phase II clinical trials[110,120,121]
Tivantinib	MET	Inhibited growth in multiple cancer cell lines *via* MET targeting as well as inhibition of microtubule formation[122]
E7050	MET, VEGFR	Inhibited growth in xenograft models of lung, gastric and pancreatic cancer[123]
PF-04217903	MET	Inhibited growth and metastasis of pancreatic neuroendocrine tumors[124]
SU11274	MET	Inhibited growth and proliferation in colon cancer cell lines[125]
T-1840383	MET, VEGFR	Inhibited tumor growth in a variety of murine xenograft models[126]

MET: Mesenchymal-epithelial transition factor; ALK: Anaplastic lymphoma kinase; NSCLC: Non-small cell lung carcinoma; VEGFR: Vascular endothelial growth factor receptor.

effectors. Unfortunately, dysregulated HGF-MET signaling is implicated in multiple oncologic mechanisms, including tumor growth, invasion and chemoresistance. Not surprisingly, clinical studies have consistently revealed MET overexpression as a negative prognostic indicator in a wide variety of malignancies.

HGF-MET signaling mediates mesenchymal-cell-mediated mitogenic support to developing tumor cell populations. MET activity enhances ECM degradation and integrin-mediated adhesion. In addition to promoting mobility and invasion, this appears to confer a protective microenvironment conducive to the development of chemoresistant clones. MET signaling is a marker of cancer stem cell populations, a recently characterized subgroup of cancer cells resistant to cytotoxic therapies.

A better understanding of tumor growth signaling pathways and chemoresistant mechanisms carries the potential of immense therapeutic value, especially in aggressive tumors such as pancreatic adenocarcinoma. Strategies include targeting chemoresistant CSCs, limiting acquired resistance with combination therapy, and developing methods of biochemically dissociating tumor cells from their mitogenic microenvironments. Each of these mechanisms has been associated with HGF-MET signaling. Not surprisingly, a series of MET inhibitors and more nonspecific RTK inhibitors are currently under investigation (Table 3)[107-111]. The evidence presented makes a compelling case for further insights into HGF-MET signaling as a therapeutic target in pancreatic cancer.

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Pancreatic cancer organotypics: High throughput, preclinical models for pharmacological agent evaluation

Stacey J Coleman, Jennifer Watt, Prabhu Arumugam, Leonardo Solaini, Elisabeta Carapuca, Mohammed Ghallab, Richard P Grose, Hemant M Kocher

Stacey J Coleman, Jennifer Watt, Prabhu Arumugam, Leonardo Solaini, Elisabeta Carapuca, Mohammed Ghallab, Richard P Grose, Hemant M Kocher, Centre for Tumour Biology, Barts Cancer Institute - a CR-UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom

Jennifer Watt, Prabhu Arumugam, Leonardo Solaini, Hemant M Kocher, Barts and the London HPB Centre, The Royal London Hospital, Barts Health NHS Trust, London EC1M 6BQ, United Kingdom

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Correspondence to: Hemant M Kocher, MS, MD, FRCS, Centre for Tumour Biology, Barts Cancer Institute - a CR-UK Centre of Excellence, Queen Mary University of London, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom. h.kocher@qmul.ac.uk
Telephone: +44-20-78823579 Fax: +44-20-78823884
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Abstract

Pancreatic cancer carries a terrible prognosis, as the fourth most common cause of cancer death in the Western world. There is clearly a need for new therapies to treat this disease. One of the reasons no effective treatment has been developed in the past decade may in part, be explained by the diverse influences exerted by the tumour microenvironment. The tumour stroma cross-talk in pancreatic cancer can influence chemotherapy delivery and response rate. Thus, appropriate preclinical *in vitro* models which can bridge simple 2D *in vitro* cell based assays and complex *in vivo* models are required to understand the biology of pancreatic cancer. Here we discuss the evolution of 3D organotypic models, which recapitulare the morphological and functional features of pancreatic ductal adenocarcinoma (PDAC). Organotypic cultures are a valid high throughput preclinical *in vitro* model that maybe a useful tool to help establish new therapies for PDAC. A huge advantage of the organotypic model system is that any component of the model can be easily modulated in a short timeframe. This allows new therapies that can target the cancer, the stromal compartment or both to be tested in a model that mirrors the *in vivo* situation. A major challenge for the future is to expand the cellular composition of the organotypic model to further develop a system that mimics the PDAC environment more precisely. We discuss how this challenge is being met to increase our understanding of this terrible disease and develop novel therapies that can improve the prognosis for patients.

Core tip: Pancreatic cancer carries a terrible prognosis, as the fourth most common cause of cancer death in the Western world. One of the reasons no effective treatment has been developed in the past decade may in part, be explained by the influences exerted by the tumour microenvironment. The tumour stroma cross-talk in pancreatic cancer can influence chemotherapy delivery and response rate. Organotypic models of pancreatic cancer allow new therapies that can target the cancer, the stromal compartment or both to be tested in a model that mirrors the *in vivo* situation and can help improve patient prognosis.

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PANCREATIC CANCER

Pancreatic cancer has one of the highest mortality rates among malignancies, and is the fourth most common cause of cancer death in the Western world[1,2]. With an overall 5-year survival rate of 6% and median survival of less than six months, pancreatic ductal adenocarcinoma (PDAC) carries one of the bleakest prognoses in all of medicine. Surgery offers the only hope of a possible cure for patients; however even of those 10% of patients eligible for curative resection, only 21% will survive to five years[3]. This is due to the fact that, at diagnosis, distant metastases are common[4]. Clearly there is an urgent need for therapies for PDAC. One of the possible reasons that targeted therapies fail to improve the prognosis of patients with PDAC may, in part, be explained by the diverse influences exerted by the tumour microenvironment. Delineating the signalling networks within the tumour microenvironment, may help to explain the huge discrepancy between relative success and effectiveness of therapies in preclinical assay (predominately 2D cell based assays and xenograft mouse models) and their abject failure in human PDAC.

Many epithelial malignancies, including breast, prostate, skin and pancreatic cancers, often exhibit a significant stromal reaction around the tumour cells[5-9]. Once thought to be a bystander, it is becoming increasingly evident that the stroma not only functions as a mechanical barrier but also constitutes a dynamic compartment that is critically involved in the process of tumour formation, progression, invasion, and metastasis[10,11]. In particular, PDAC shows the most prominent stromal reaction or "desmoplasia" (defined as proliferation of fibrotic tissue with an altered ECM which contributes to tumour growth and metastasis) (Figure 1)[12]. This surrounding tumour environment is an highly heterogeneous and complex mixture of cells from different lineages; fibroblasts, pancreatic stellate cells, smooth muscle cells, immune, inflammatory, neural, adipose and endothelial cells[13-16].

The high proportion of stromal cells in pancreatic cancer (up to 80% of the tumour volume[17]) is associated with overexpression of a number of paracrine and autocrine signalling factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor β (TGFβ), insulin-like growth factor I (IGF- I), fibroblast growth factor (FGF) and their respective receptors as well as secretion of Matrix metalloproteinases (MMPS) and proteases which serve to fuel pancreatic cancer proliferation, metastasis and invasion[18-22]. In turn, pancreatic cancer cells secrete growth factors such as FGFs, TGFβ, IGF and platelet derived growth factor (PDGF)[23]. This interaction between cancer cells and stroma leads to altered transcrip-

tion in stromal components, such as fibroblasts and inflammatory cells, promoting cancer cell motility and resistance to hypoxia. The net result is an unique tumour micro-environment, where tumour cells become inaccessible to chemotherapy and metastasise readily, leading to poor chemotherapy response rate[17].

These studies have highlighted the importance of stroma-cancer cross-talk. Thus, just studying pancreatic cancer cells without any stromal representation does not reflect accurately the *in vivo* situation. Cells grown on 2D tissue culture plates or in Transwell™ inserts differ in their morphology, differentiation and cell-cell and cell-matrix interactions compared to cells *in vivo*[24,25]. There is a need for physiologically relevant *in vitro* model systems that allow us to investigate and interrogate cancer and stromal cell behaviour and their interactions. Thus, 3D organotypic models are an invaluable research tool[26].

MODELLING PDAC

In vitro (2D) studies of tumour stroma interactions in PDAC

Improved understanding of the mechanisms that mediate epithelial-stromal interactions in PDAC is now possible due to the isolation, and *in vitro* culture, of pancreatic stellate cells (PSC), the key cells driving the desmoplastic reaction[21]. In the healthy pancreas, PSCs make up 4%-7% of all pancreatic cell types and exist in a quiescent state[27]. Quiescent PSCs are characterised by lipid droplets rich in vitamin A, resembling hepatic stellate cells (HSCs) first described by in the 19th century[28]. They express desmin and glial fibrillary acid protein (GFAP) marker which serve to distinguish them from pancreatic fibroblasts[29]. In acute and chronic inflammatory conditions, PSCs are activated. This is characterised a loss of fat droplets, expression of α-smooth muscle actin (αSMA), and an increased synthesis and secretion of several ECM proteins such as fibronectin, laminin and collagen type I and III[27,30,31].

The isolation and immortalisation of PSCs from human and rat pancreas has provided an additional tool for studying PSC activation and can overcome the limitations of culturing primary stellate cells. While immortalised stellate cells have provided a valuable tool in the study of PSC function, it is important to validate findings using primary PSCs[14]. PSCs have been immortalised using either SV40 large T antigen or human telomerase in human PSCs as we have previously successfully done in our laboratory[24,32-38]. Immortalised PSCs display an activated phenotype in 2D culture. Importantly PSC cell line is comparable to activated PSCs, which include expression of αSMA and ECM proteins. Importantly, expression profiling of primary and PSC cell lines have shown only a few differences, with differential differences expression of ECM proteins, cytokines and integrins[37]. In addition, both immortalised and primary PSCs respond to TGF-β or PDGF in a similar manner[33]. Thus primary and immortalised PSCs have facilitated for the dissection of
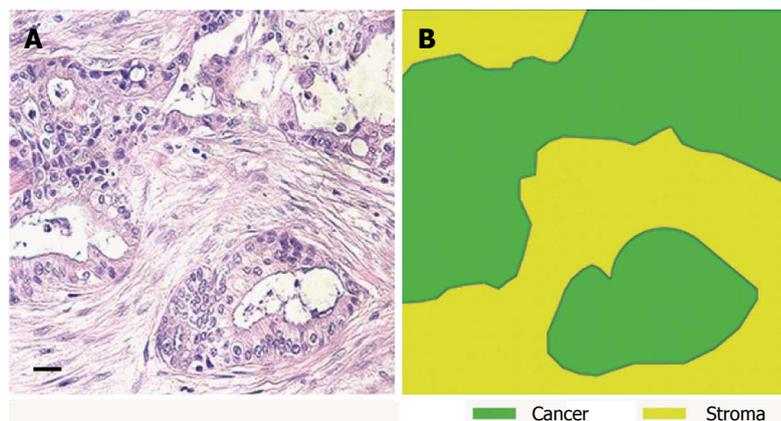
Figure 1 Human pancreatic ductal adenocarcinoma has a dense desmoplastic stromal component. A: HE of human pancreatic cancer shows an area of invasive tumour; B: Stromal and epithelial components of the tumour are highlighted from figure A (scale bar 100 μm).

important cross-talk between PSCs and pancreatic cancer cells and are an important source to explore the tumour promoting aspects of tumour myofibroblasts in PDAC[16].

The bidirectional interaction between PSCs and pancreatic cancer cells has been studied using co-culture or well-established 2D *in vitro* assays such as wound assays or Transwell™ inserts to study migration[39]. Co-culturing of PSCs and pancreatic cancer cells showed that PSCs can increase the proliferation and migration of pancreatic cancer cells, while inhibiting apoptosis by the release of several cytokines and growth factors. Similarly, culturing PSCs in the conditioned medium of pancreatic cancer cells increases the proliferation, matrix synthesis and motility of PSCs, most likely *via* FGF-2, PDGF and TGF-β[22,40].

The desmoplasia in PDAC is believed to have a detrimental effect on the successful response to chemotherapy and radiotherapy[40,41]. *In vitro* experiments have shown that PSCs can increase the stem cell characteristic of pancreatic cancer cells, a possible mechanism of resistance to therapy[42]. Furthermore, in areas of the tumour that are hypoxic as a result of hypovascularity and profuse stroma provides a micro-environment in which pancreatic cancer cells thrive[43]. *In vitro* studies have shown that, co-culturing PSCs and pancreatic cancer cells under hypoxic conditions, PSCs are able to influence PCC invasion more strongly than in normoxic conditions[44]. Thus, pharmacological targeting of PSCs is an attractive option in treating PDAC.

Role of the stroma in PDAC-in vivo studies
Animal models, such as xenografts, orthotopic grafts or genetically engineered mice (GEM), have validated many *in vitro* findings. Early subcutaneous mouse models, in which PSCs and pancreatic cancer cells were injected into the flanks of immunocompromised mice, demonstratied that, in the presence of PSCs, pancreatic cancer cell proliferation increased and tumours formed more rapidly than when pancreatic cancer cells were injected alone[22]. Apte and colleagues showed that injection of pancreatic cancer cells (MiaPaCa-2 and AsPC-1 cell lines), together with primary human PSC into the mouse pancreas was able to stimulate fibrosis, tumour growth and metastasis[40]. More recently, sex mismatch studies

(injection of male PSCs and female pancreatic cancer cells into the pancreas of female mice), have shown that Y chromosome positive PSCs are able to migrate through blood vessels, together with cancer cells, localising to distant sites, such as the liver and diaphragm, where they are able to facilitate seeding, survival and growth of pancreatic cancer cells[45].

The development of genetically engineered mouse (GEM) models of PDAC has provided the most physiologically relevant model that closely mimics the situation in human cancer. Most of the GEM models of PDAC are based on the conditional, pancreas-specific, expression of the Kras oncogene ($KRAS^{G12D}$), present in 90% of human PDAC cases[46], this is facilitated by expressing Cre recombinase under the control of the embryonic pancreas lineage determining transcription factor Pdx-1 or Ptf1/p48 ("KC" mice). KC mice develop pancreatic tumours ranging from precursor pancreatic intra-ductal neoplasms (PanINs) to fully invasive and metastatic disease[47,48], albeit with a long latency period of up to a year. These KC mice have been crossed with mice harbouring several additional mutations, to investigate their contribution to the rapid progression to PDAC. GEM models of PDAC have been developed with activating mutations in TGFβ receptor and/or inactivation of tumoral suppressors such as p53 ("KPC" mice), INK4A/ARF and Smad4, which are the most common PDAC drivers[49]. There are several excellent reviews on the various GEM models that have been developed for studying the development of PDAC[50-52]. The generation of complex allele combinations together with the latency period involved in the development of tumour makes these models inherently expensive. Further criticism against GEM models of PDAC has focused on the multi-focality of their PDAC, involvement of whole pancreas with tumours, histological variants commonly observed, presence of tumours in other organs as well as genetic homogeneity; features missing in the human PDAC[53]. Thus, 3D organotypic models may be an attractive option as a preclinical tool, bridging the gap between traditional 2D cell culture assays and the complex GEM models.

Organotypic models used in other cancers
The idea of recapitulating the physiomimetic 3D envi-
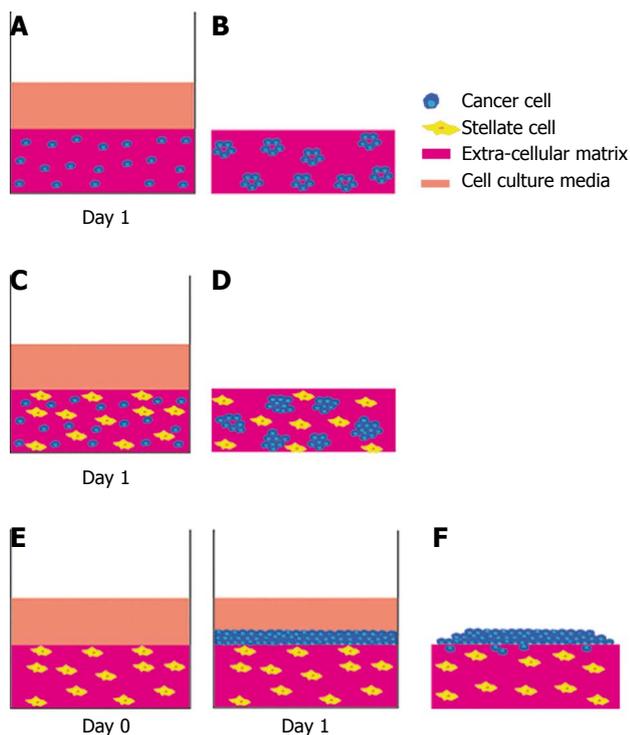
Figure 2 Submerged organotypic culture models used to investigate pancreatic ductal adenocarcinoma. A: Cancer cells were embedded in the extracellular matrix mixture before it was allowed to polymerise. These cells were then fed with culture media placed on top of the gel; B: Representative configuration of cells within the gel after 7 d of culture. The cancer cells forming duct-like structures within the gel. This model mimics the behaviour of invading cancer cells; C, D: Show the same model with both pancreatic stellate cells and cancer cells embedded within the extracellular matrix gel. Using this model the interaction between stellate cells and invading cancer cells can be examined; E: Stellate cells embedded in the gel prior to polymerisation, with cancer cells seeded on top of the gel 24 h later. In this model cancer cell invasion can be analysed in the presence of pancreatic stellate cells (F). Representatice HE of these organotypic models are reviewed in Froeling *et al*[26].

ronment started in the 1960's growing 3D tissue explants in tissue culture media (organ cultures). Organ cultures of neural tissue explants are perhaps the best established model[54]. These models are still being used to study the basis of neurological diseases and injuries[55]. Organ cultures are also used in the study of cardiovascular function[56], angiogenesis[57], thymus[58], skin[59], bone[60], and urogenital tissues[61].

In cancer research, there has been an abundance of evidence suggesting that 3D models are superior to the conventional 2D culture in plastic flasks. However, current preclinical research still relies heavily on the latter[62]. From the simplest form: the "monotypic" cell model, comprising just one epithelial cell type, 3D co cultures have progressively evolved to contain multiple cell types, thus enabling study of their respective contributions[63]. An early example was the the "skin equivalent", achieved by culturing keratinocytes either on de-epidermalised dermis or on collagen gels embedded with dermal fibroblasts[64,65].

The success of pioneering studies with breast epithelial cells cultured in, or on, a reconstituted basement membrane (*e.g.*, Matrigel) undergoing glandular differentiation forming with apico-basal polarity and a central hollow lumen[66], have led to similar experiments for the liver, salivary gland, bone, lung, skin, intestine, kidney and thyroid glands[64,67-71]. The choice of cell source and ECM is critical in developing a representative model. For example, human luminal epithelial cells, grown in laminin rich basement membrane analogue (Matrigel) form acini[72]; however when grown in collagen I, these same cells show an altered integrin profile and abnormal polarity[73].

These 3D models have increased our understanding of how cells perceive biochemical and physical cues from the surrounding microenvironment[74]. For example, β1 integrin is expressed in normal breast epithelial cells but is lost when cells transform into a malignant phenotype. Re-expression of β1 integrin in 3D matrices induces the reversion of the tumor phenotype by allowing the malignant cells to differentiate into glands[75].

The incorporation of tissue specific stromal cells is critical for approximation to the *in vivo* condition. Thus, the isolation and availability of human PSCs hase been critical to the development of PDAC organotypic cultures[26].

Pancreatic cancer organotypics

Pancreatic cancer cell lines and normal pancreatic ductal epithelial cells (HPDE) previously have been cultured on type I glycosaminoglycan scaffolds and in collagen type I or Matrigel. Given only epithelial cells were in these models, the effect of the stroma on tumour cell behaviour was absent[76-78]. However, these studies were able to show that pancreatic cancer cells embedded into Matrigel formed spheroids with a distinct morphology and loss of apico-basal polarity as compared to culturing in 2D[76].

The introduction of stromal cells in PDAC 3D organotypic cultures was first demonstrated by our laboratory[24]. Depending on the hypothesis being explored, the flexible 3D models of PDAC can be set up distinctly. Pancreatic cancer cells can be embedded into the ECM gel consisting of collagen and Matrigel to simulate cells that have already invaded into the stroma However, in order to understand the influence of PSCs on the behaviour of invaded pancreatic cancer cells these cells can be embedded in an ECM gel together with cancer cells (Figure 2).

Submerged ECM gels (when pancreatic cancer cells are grown on top of the gel and PSCs are embedded) are designed to model the early events in tumour progression. When pancreatic cancer cells are cultured on top of this model, they form luminal structures that resemble ducts (Figure 3). Using this model, we have shown that PSCs induce Ezrin translocation from the apical to the basal compartment of the cells is an early event in pancreatic cancer cell invasion[24,79]. This phenomenon has been validated across a range of human gastro-intestinal tumours[80,81]. Finally, in order to study the invasion of pancreatic cancer cells in the 3D model, the submerged
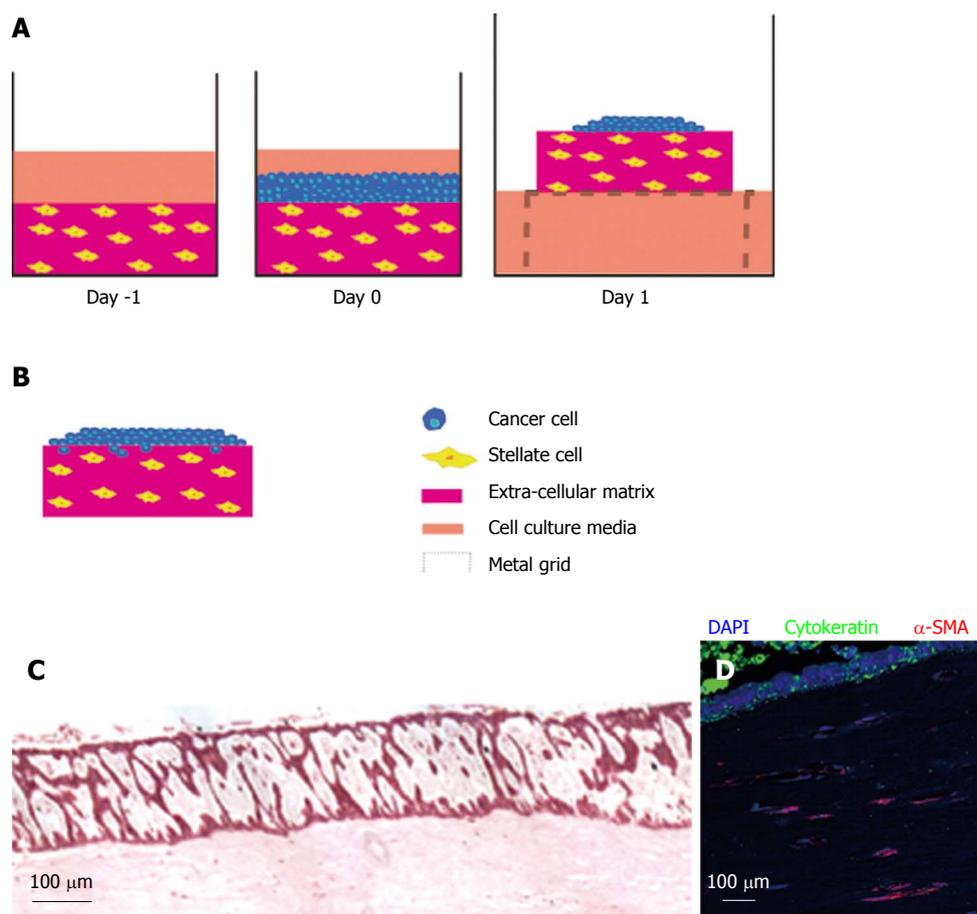
Figure 3 Raised organotypic model of pancreatic ductal adenocarcinoma with embedded pancreatic stellate cells. A: Extracellular matrix gels containing stellate cells were polymerised in 24-well plates before cancer cells were seeded on top and allowed to attach. These gels were then raised onto metal grids and fed from below creating a chemotactic gradient. Cells were cultured for up to 14 d; B: Illustration of cancer cells in a raised model containing stellate cells showing proliferation and invasion into the gel; C: HE section of a raised gel containing stellate cells with cancer cells seeded on top; D: Immunofluorscence in the same gel showing strong cytokeratin expression in the cancer cells and α-smooth muscle actin (α-SMA) expression in the embedded stellate cells.

culture system can be raised upon a grid ('air-liquid' model) and fed from underneath, creating a gradient that stimulates pancreatic cancer cells to invade while at the same time recapitulates cancer-stellate cell interaction *in vivo* (Figure 4).

Using the air liquid 3D model we have shown that the presence of PSCs leads to a significant increase, and altered sub-cellular distribution, of β-catenin in pancreatic cancer cells. Treating these 3D co cultures with All Trans Retinoic Acid (ATRA, which renders PSC quiescent) dampens Wnt-β catenin signalling resulting in reduced pancreatic cancer invasion[16]. Importantly, these results were confirmed *in vivo*, whereby treating KPC mice with ATRA led to disruption lead to disruption of the activated stroma and increase in apoptosis of tumour cells. These sets of observations validate the use of the organotypic model as a tool to assess new therapies in PDAC.

3D organotypic models provide a perfect intermediate between 2D cultures and GEM. Use of distinct cell types in these co-culture allows assessment of changes in signalling cascades and molecular targets resulting from cancer-stroma cross-talk in the absence of noise from other stromal elements present *in vivo*. Thus the relative

contribution of each cell type in the complex microinvrionment can be assessed. Using this approach Kadaba *et al*[14] isolated cancer cells from organotypic models of various organ including pancreas, skin and oesophagus after the cancer cells were exposed in 3D to their respective stromal cells (Figure 5). They demonstrated that cancer cell stromal interactions significantly alter proliferation, cell cycle, cell movement, cell signalling and inflammatory response in addition to changing stiffness in the ECM gel. Importantly, changes in stiffness of ECM gels was particularly prominent as the proportion of PSC in the ECM gel increased, a finding highly pertinent to drug delivery and perfusion in PDAC[41]. This study also highlighted the possible need for multidrug targeting or use of pleiotropic agents in PDAC therapy.

Despite the importance of multiple pathways in PDAC, the proto-oncogene Src has been heralded as a potential single molecular therapeutic target[82]. The conundrum of promise of Src inhibitors in combination with chemotherapy *in vitro* and the *in vivo* reduction of metastasis in KPC mice by 50%, was explored in organotypic cultures[82]. Using fluorescence lifetime imaging microscopy (FILM) to measure fluorescence resonance
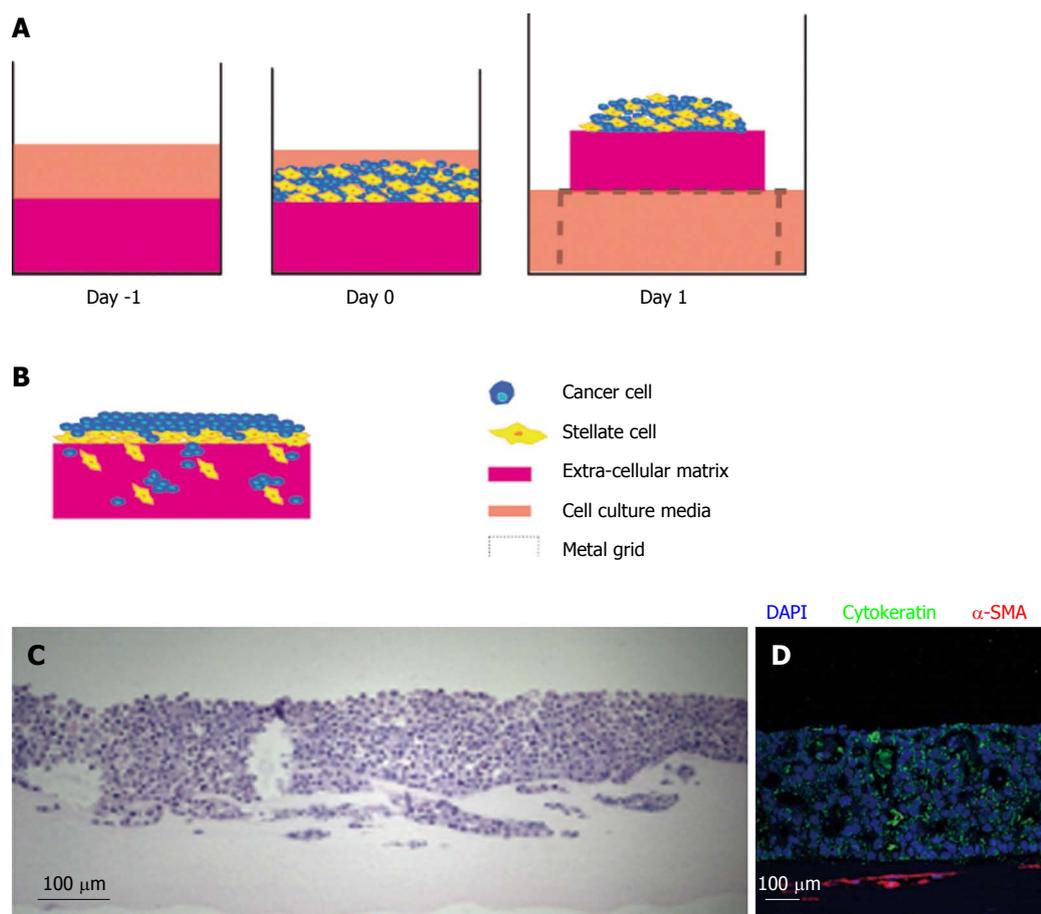
Figure 4 Raised organotypic model of pancreatic ductal adenocarcinoma with cancer cells and pancreatic stellate cell interaction. A: Extracellular matrix gels containing were polymerised in 24-well plates before cancer stellate cells were seeded on top and allowed to interact and attach. These gels are then raised onto metal grids and fed from below creating a chemotactic gradient. Organotypic models were cultured for up to 14 d; B: Illustration of cancer cells and stellate cells in a raised model showing increased proliferation and invasion of both stellate and cancer cells into the gel; C: HE section of a raised gel with cancer cells and pancreatic stellate cells seeded on top; D: Immunofluorescence in the same gel showing strong cytokeratin expression in the cancer cells and α-smooth muscle actin (α-SMA) expression in the stellate cells which form a layer below the cancer cells.

energy transfer (FRET) an ECFP-YFP Src reporter, in PDAC cells in organotypic cultures Anderson and colleagues investigated the influence of tumour microenvironment on Dasatinib delivery in PDAC[83]. In organotypic PDAC models with cancer cells expressing the Src biosensor cultured on top of an ECM gel with embedded primary human fibroblasts, they were able to show quantitatively that the microenvironment contribution to poor drug delivery to tumour cells is dependent on distance of cells from the invasive edge. This was validated in subcutaneous *in vivo* models due to the limitations of microscopy techniques precluding orthotopic or GEM models. This study demonstrated the adaptability of the organotypic model as powerful tool to address hypotheses at the molecular level in a complex microenvironment.

Future applications and challenges

3D organotypic models that mimic the morphological and functional features of their *in vivo* parental tissues have potential for bridging the gap between cell-based discovery research and animal models[84,85]. A huge advantage of the organotypic system is that any component of the model can readily be modulated in a short time-frame.

For example, the matrix composition can be altered to reflect the *in vivo* situation. The increase in ECM stiffness exerts elevated force on transformed cells increasing cellular response and resulting in increased tumour growth, survival and motility[14,86].

The relative paucity of primary stellate cells to conduct all the experiments in sufficient replicates lead us to generate a mini organotypic culture system (Figure 6) which give comparable results to the conventional "air liquid" co culture model[14]. Additional cell types can be titrated in such as stellate cells[14] or endothelial cells (Di Maggio, unpublished observations). For example, to assess the role of stroma on angiogenesis, in oesophageal cancer endothelial cells on a 2D monolayer have been cultured with fibroblast and cancer cells embedded in a collagen gel layered on top[87]. Elsewhere investigation of the role of macrophages in malignant growth of human squamous cell carcinoma has been investigated in organotypic cultures[88]. Immune response and inflammation play an important role in the desmoplastic reaction and inflammation is thought to activate pancreatic stellate cells[13,89].

Therapeutic agents such as chemotherapy (Gemen-
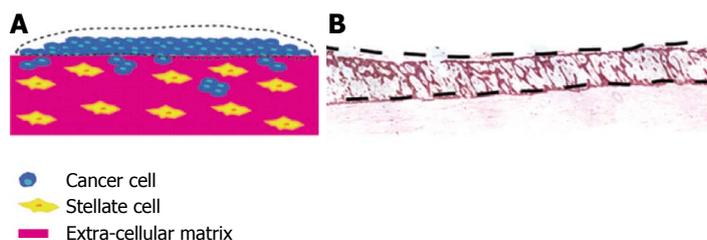
Figure 5 Use of organotypic model to isolate cell types grown together by laser microdisection. By using the raised organotypic model with pancreatic stellate cells embedded within the gel (Figure 4B) the sellate and cancer cells are kept separate. A, B: Laser microdissection of the cancer cells can then be performed to allow analysis of cancer cells grown in the presence of pancreatic stellate cells. Scale bar 100 μm.
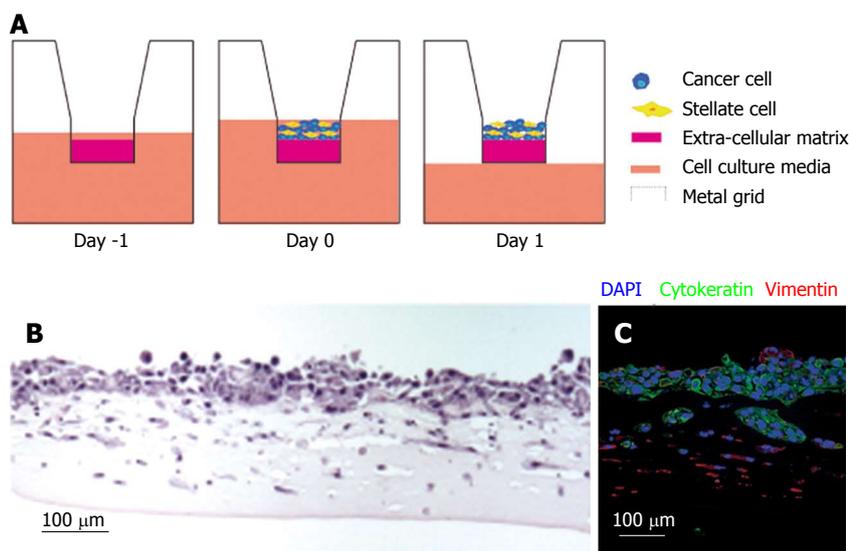
Figure 6 Mini-organotypic model of pancreatic ductal adenocarcinoma. Extra cellular matrix gel is polymerised within the insert of a standard migration assay plate. A: Cancer and pancreatic stellate cells are seeded on top of the gel and allowed to attach. The media is then removed and then replaced only in the bottom of the well to again create a chemotactic gradient cells are cultured for 7-10 d; B: HE image demonstrating a similar pattern of cell proliferation and invasion is seen, as in the raised model; C: Immunofluorscence in the same gel showing strong cytokeratin expression in the cancer cells and vimentin expression in the stellate cells which form a layer below the cancer cells and invade into the gel ahead of cancer cells.

zitdis and Carapuca and Ghallab, unpublished observations), small molecules[90] or RNAi (Arumugam and Watt, unpublished observations) can be tested in these organotypic cultures. The best dosage and regimen can then be taken in small animals thus reducing animal usage[16,83]. Examples from other related fields include testing Met-kinase inhibitor or COX-2 inhibitor in skin cancer models[91], tyrosine kinase inhibitors for breast cancers[92] and Eps8 and HAX1 or β6 integrin[93] RNAi in cancer cells prior to their incorporation into organotypic cultures to assess the effects on cell invasion.

Finally, many PDAC patients present very late with their disease when metastasis have already occurred. Thus, treating PDAC cells immediately after seeding in a 3D environment does not reflect the true clinical setting as tumours are well established at the time of patient treatment. We currently are investigating the effect of treating organotypic models once they are established and invasion of PDAC and/or stromal cells has begun. It is likely this would give a better understanding of the treatment regimen that is required when novel therapies emerge into a preclinical setting.

CONCLUSION

Organotypic culture models are valuable tools for study-ing the mechanisms of pancreatic cancer, providing an easily manipulated system in which specific questions can be addressed, thus facilitating the translation of basic science to the clinic. Allowing manipulation of cell types, matrix composition, and exogenous therapies, these physiologically relevant model systems are reproducible, experimentally flexible and offer targeted high-throughput platforms. Although the organotypic model provides a physiologically relevant means to study the tumour stroma interactions and the use of new therapies to target the cross talk, it remains a simplified representation of the complex *in vivo* situation and it still remains critical to test new therapies in orthotopic or transgenic models of the disease. However, the use of the organotypic model as a preclinical tool is becoming increasingly important and our group, as well as others, are modulating the 3D cultures to recapture other important aspects of the tumour microenvironment that can influence cancer cell behaviour. Thus, 3D organotypic models have potential for bridging the gap between cell based discovery and complex animal models. By providing an environment in which cell behaviour and novel treatment options can be investigated in an easily reproducible and controlled manner, these models more precisely mimic pancreatic cancer, thus providing a major contribution to preclinical drug and therapeutic discovery.

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2014 ADVANCES IN PANCREATIC CANCER

hENT1 expression is predictive of gemcitabine outcome in pancreatic cancer: A systematic review

Stina Nordh, Daniel Ansari, Roland Andersson

Stina Nordh, Daniel Ansari, Roland Andersson, Department of Surgery, Clinical Sciences Lund, Lund University and Skåne University Hospital, SE-221 85 Lund, Sweden
Author contributions: Nordh S performed the literature search; Nordh S and Ansari D were involved in data analysis and manuscript writing; Andersson R designed the study and revised the manuscript; all authors read and approved the final manuscript.
Correspondence to: Roland Andersson, MD, PhD, Department of Surgery, Clinical Sciences Lund, Lund University and Skåne University Hospital, Paradisgatan 2, SE-221 85 Lund, Sweden. roland.andersson@med.lu.se
Telephone: +46-46-172359
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Abstract

High human equilibrative nucleoside transporter 1 (hENT1)-expression has shown a survival benefit in pancreatic cancer patients treated with gemcitabine in several studies. The aim of this systematic review was to summarize the results and try to assess the predictive value of hENT1 for determining gemcitabine outcome in pancreatic cancer. Relevant articles were obtained from PubMed, Embase and Cochrane databases. Studies evaluating hENT1-expression in pancreatic tumor cells from patients treated with gemcitabine were selected. Outcome measures were overall survival, disease-free survival (DFS), toxicity and response rate. The database searches identified 10 studies that met the eligibility criteria, and a total of 855 patients were included. Nine of 10 studies showed a statistically significant longer overall survival in univariate analyses in patients with high hENT1-expression compared to those with low expression. In the 7 studies that reported DFS as an outcome measure, 6 had statistically longer DFS in the high hENT1 groups. Both toxicity and response rate were reported in only 2 articles and it was therefore hard to draw any major conclusions. This review provides evidence that hENT1 is a predictive marker for pancreatic cancer patients treated with gemcitabine. Some limitations of the review have to be taken into consideration, the majority of the included studies had a retrospective design, and there was no standardized scoring protocol for hENT1-expression.

Key words: Pancreatic cancer; Gemcitabine; hENT1; Predictive; Survival

Core tip: Human equilibrative nucleoside transporter 1 is a predictive marker for pancreatic cancer patients treated with gemcitabine.

INTRODUCTION

Gemcitabine is the standard chemotherapy treatment for pancreatic cancer[1-3], but its efficacy is limited; only 15% of patients with advanced pancreatic cancer[4] and up to 30% in general[5] can be expected to respond to treatment. Gemcitabine is hydrophilic and therefore passive diffusion through hydrophobic cellular membranes is slow[1]. Permeation through the membranes requires specialized membrane transporters[1,3], and human equilibrative nucleoside transporter 1 (hENT1) is the most important for gemcitabine[6,7]. Because gemcitabine is a prodrug, it has to be phosphorylated after intracellular uptake[1] in order to have a cytotoxic effect[6]. This rate-limiting step is carried out by the enzyme deoxycytidine kinase (dCK)[8].

Recent research has revealed that differences in the

expression of genes, including hENT1[9,10] and enzymes involved with gemcitabine metabolism, such as dCK, may be predictors of the efficacy of gemcitabine treatment for pancreatic cancer[11]. Several studies have indicated that high expression of hENT1 is associated with longer overall survival (OS) and longer disease-free survival (DFS)[9,10,12].

The aim of this review was to evaluate and summarize the potential predictive value of hENT1 expression in pancreatic tumor cells in patients treated with gemcitabine.

STUDY SELECTION

To identify all relevant English-language articles published from 1966 to March 2013, a computerized search of PubMed, Embase and Cochrane databases was performed. The following search terms were used: (hENT1 OR nucleoside transporter), (gemcitabine OR gemzar), (pancreatic OR pancreas), (cancer OR adenocarcinoma OR neoplasm). The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)[13] was used as a guideline for the processing and reporting of the results. The initial search yielded 230 publications (54 in PubMed, 1 in the Cochrane database, 175 in Embase). To find studies that might have been missing in the database search, a manual search was made by reading through reference lists of relevant articles and systematic reviews. The results of the search and the selection of studies are shown in Figure 1. The quality of the included articles was assessed using the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK)[14].

ELIGIBILITY CRITERIA

For inclusion in this systematic review, the following criteria had to be met: retrospective or prospective studies of patients with pancreatic cancer, all stages, treated with gemcitabine with or without additional radiation; the expression of hENT1 had to be reported and related to patient outcome; and the articles had to be available in full text and published in English. Exclusion criteria were conference abstracts, overlapping patient cohorts and studies in which the relevant outcomes of interest were not addressed.

DATA EXTRACTION

A data extraction form was completed before the extraction process began. The form was reviewed by a second author (RA) to ensure that all relevant information was being extracted. The data extraction was done by a single reviewer (SN) and the following data were extracted from each study: publication details [author(s), date of publication, location, study center], study design, population details (age, sex, pT-stage, pN-stage), patient number, type of intervention (dose, schedule, duration), method to determine hENT1-expression, hENT1-scoring, primary

and secondary outcome measurements, and results correlated with hENT1-expression.

OUTCOMES OF INTEREST AND DEFINITION

The primary outcome measures were OS and DFS correlated with hENT1 expression. Secondary outcome measures were toxicity according to the Common Toxicity Criteria (http://www.eortc.be/services/doc/ctc/), and response rate according to RECIST[15] criteria.

LITERATURE SEARCH

The search identified 230 references in the 3 databases. Of these, 120 were excluded after identification of duplicates and exclusion based on irrelevant titles. Abstracts from 110 articles were screened and 75 were excluded. The main reasons for exclusion were: nonclinical trials (such as review articles), hENT1-expression was not being assessed, and irrelevant outcome measurements. The remaining 35 studies were retrieved for further assessment. Of these, 25 references were excluded for the following reasons: they were conference abstracts and not full-text articles; hENT1-subgroups were measured[16,17]; hENT1 was evaluated as a prognostic factor rather than a predictive factor of gemcitabine treatment[18]; there were overlapping patient populations[19]; and too small sample size/case reports[20]. An additional 5 article abstracts were screened for eligibility after identification in a manual search of reference lists of relevant articles. All of these were excluded based on irrelevance. In total, 10 studies fulfilled our inclusion criteria and were included in this systemic review.

CHARACTERISTICS OF SELECTED STUDIES

The included articles were published between 2004 and 2012. They originated from Belgium (2 studies)[1,3], Canada (one study)[9], United States (one study)[12] and Japan (5 studies)[2,8,21-23]. The 5 studies from Japan originated from 5 different universities, Kyushu, Osaka, Yokohama, Mie and Hiroshima. One author had published 2 articles[1,3], for one of these the patient population was recruited from 2 centers, while for the other the patient population was recruited from 5 centers. The potential bias of overlapping patient populations was therefore small, but must nevertheless be taken into consideration in the analysis of the results.

Nine of 10 studies were retrospective[1-3,8-10,21-23] and one was a *post hoc* analysis of a randomized controlled study[12]. The 10 studies involved a total of 855 patients and the sample size varied from 21 to 234 (Tables 1 and 2).

Four studies[1,8,10,12] used parallel groups, while the remainder were single-arm studies. The treatment protocols, which differed between the studies, included ad-
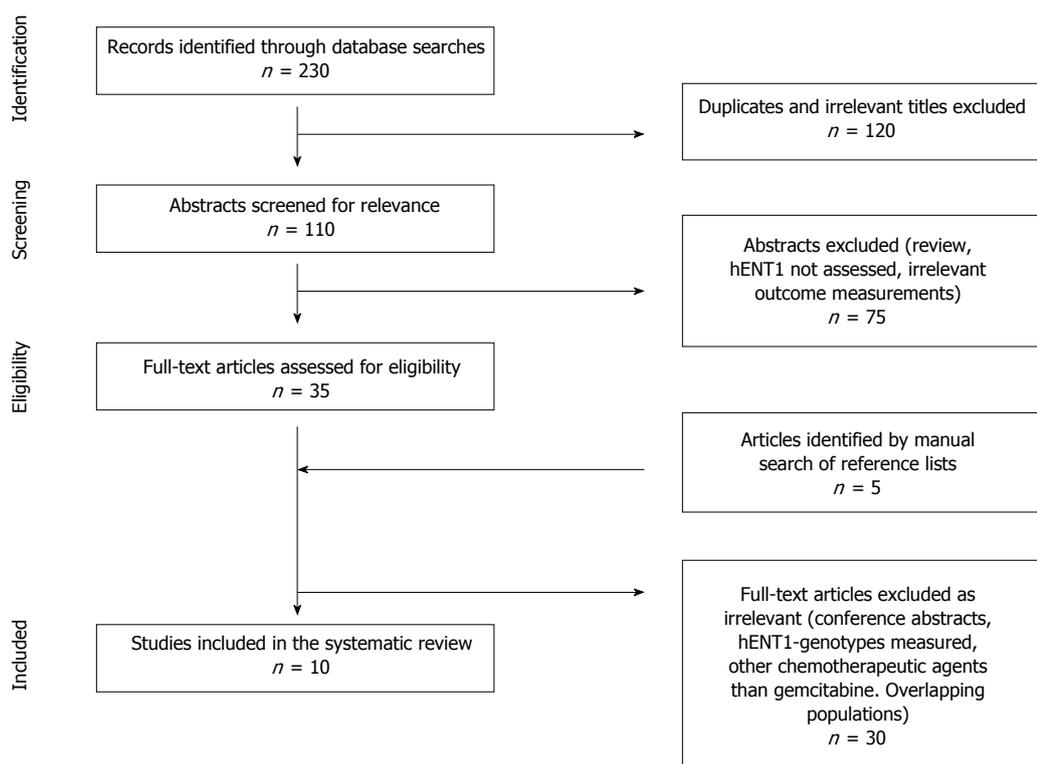
Figure 1 Flowchart showing article selection process.

Table 1 Characteristics of the identified studies

Ref.	Year of publication	Country	Inclusion period	No. of patients	Study design	Follow-up median (95%CI), mo
Spratlin *et al*[9]	2004	Canada	1998–2002	21	RS	NR
Giovannetti *et al*[10]	2006	Italy	2001–2004	102[1]	RS	11.2 (0.4–32.1)
Farrell *et al*[12]	2009	United States	1998–2002	91	*Post hoc*[2]	NR
Maréchal *et al*[3]	2009	Belgium	2000–2003	45	RS	21.9 (3.3–107.4)
Fujita *et al*[8]	2010	Japan	1992–2007	70	RS	15.7 (0.5–114)
Maréchal *et al*[1]	2012	Belgium	1996–2009	234	RS	55.7 (46.4–61.2)
Kawada *et al*[2]	2012	Japan	2002–2007	63	RS	31
Morinaga *et al*[21]	2012	Japan	2006–2008	27	RS	NR
Murata *et al*[22]	2012	Japan	2005–2010	93	RS	15 (3.5–57.2)
Nakagawa *et al*[23]	2012	Japan	2002–2011	109	RS	39.7 (2–122)
				Total: 855		

[1]$n = 81$ with complete hENT1; [2]*Post hoc* analysis of randomized controlled trial. NR: Not reported; RS: Retrospective.

juvant gemcitabine monotherapy, palliative gemcitabine treatment, neoadjuvant gemcitabine chemotherapy, adjuvant gemcitabine chemotherapy + radiation, neoadjuvant gemcitabine + radiation (and adjuvant 5-fluorouracil), resection only and neoadjuvant gemcitabine-based chemoradiation + adjuvant gemcitabine (Table 2). All protocols were based on gemcitabine treatment and resection of the tumor.

hENT1 EXPRESSION

To quantify hENT1-expression, 8 studies used immunohistochemistry (IHC) and 2 used reverse transcription polymerase chain reaction. Grading of the expression differed between the studies, as is described in more de-

tail in Table 3. The majority of the studies dichotomized the expression in high/positive *vs* low/negative hENT1 expression. There were no standardized scoring procedures available.

OVERALL SURVIVAL

Nine of the 10 included studies had OS as an outcome measurement of interest (Table 4). Kawada *et al*[2] was the sole study that only reported disease-specific survival (DSS) as the primary outcome. The definition of DSS is the length of time from either the date of diagnosis or start of treatment for a specific disease (*e.g.*, pancreatic cancer), and that the patients with the disease still are alive. The difference from OS is that OS measures death

Table 2 Characteristics of the identified studies

Ref.	Age median, yr (range)	Sex m/tot *n* (%)	hENT1 method	Chemotherapy	Radiation dose Gy/(Gy/frac)	Outcome measurement	Quality REMARK
Spratlin *et al*[9]	58 (51-64)[1]	11 (52)	IHC	Pall Gem	No	OS	10
Giovannetti *et al*[10]	65 (22-83)	53 (50)	RT-PCR	Pall Gem Adj Gem	45	OS, DFS, TTP, RR	12
Farrell *et al*[12]	53/63/65[2]	45 (49)	IHC	Adj Gem	50.4	OS, DFS, Tox	17
Maréchal *et al*[3]	56 (34-83)	23 (51)	IHC	Adj Gem	40-50.4	OS, DFS, Tox	13
Fujita *et al*[8]	65 (36-86)	42 (60)	RT-PCR	Adj Gem or Resection only	No	OS, DFS	12
Maréchal *et al*[1]	NR	129 (53)	IHC	Adj Gem	50.4	OS	15
Kawada *et al*[2]	- (41-81)	33 (52)	IHC	Neo Gem Adj 5-FU	50/2	DSS	9
Morinaga *et al*[21]	64 (45-74)	17 (63)	IHC	Adj Gem	No	OS, DFS	12
Murata *et al*[22]	68 (44-87)	38 (69)	IHC	Neo Gem Adj Gem	45/2	OS, DFS, RR	13
Nakagawa *et al*[23]	67 (41-83)	52 (48)	IHC	Adj Gem + S1	No	OS, DFS	13

[1]95%CI; [2]Medians in the different hENT1 expression groups. IHC: Immunohistochemistry; RT-PCR: Reverse transcription polymerase chain reaction; Adj: Adjuvant; Neo: Neoadjuvant; Radio: Radiotherapy; Gem: Gemcitabine; Pall: Palliative; OS: Overall survival; DFS: Disease-free survival; Tox: Toxicity; TTP: Time to progression; DSS: Disease-specific survival; RR: Response rate.

from any cause, not just death from a particular disease. Survival times for the individual studies were calculated based on: diagnosis, in one study[10]; the start of gemcitabine treatment in 2 studies[9,22]; resection, in 5 studies[1,3,8,21,23]; and randomization in one study[12].

In univariate analyses, all 9 studies that had OS as an outcome measurement of interest showed a survival benefit with gemcitabine treatment and high/positive expression of hENT1 compared with patients with low/negative hENT1 expression. Multivariate analyses were conducted in 8 of the 9 studies. Seven of these identified high/positive hENT1 as an indicator of longer OS in patients with pancreatic cancer who received gemcitabine treatment. One study[8] indicated a trend towards better OS in the multivariate analysis, but this was not statistically significant (*P* = 0.2).

DISEASE-FREE SURVIVAL

In 7 studies, DFS was reported as an outcome measurement (Table 4). DFS was calculated from the same starting points as reported above for OS. Six of these studies showed a statistically significant longer DFS in univariate analyses. One study[8] was not statistically significant with regard to hENT1. Multivariate analyses were conducted in 5 studies but of these only 3[3,12,23] reported statistically significant results with longer DFS in regard to hENT1 in patients with pancreatic cancer treated with gemcitabine. Morinaga *et al*[21] and Murata *et al*[22] also performed multivariate analyses, but the results did not prove to be statistically significant with reported *P*-values ranging from 0.129 to 0.232.

TOXICITY

Only 2 studies addressed the issue of toxicity[3,12], but the numbers published were inadequate for further analy-

sis. Farrell *et al*[12] tried to find a relationship between hENT1-levels and the incidence of grade Ⅲ or higher toxicities; however no relationship was found using a logistic regression model. The analysis data were not shown in the article. Maréchal *et al*[3] reported that grade Ⅲ/Ⅳ hematological toxicities were noted in 10/45 patients and grade Ⅲ/Ⅳ nonhematological in 3/45 patients. They did not relate this finding to hENT1 expression and no further data or data analysis was shown in the article with regard to toxicity.

RESPONSE RATE

Of the 10 included studies only 2[10,22] reported the outcome measurement response rate (RR). Giovannetti *et al*[10] evaluated RR in 34/36 patients in a group of patients receiving palliative treatment with gemcitabine. Two of the patients were not evaluable because of early death and refusal. The results showed that 5 patients had a partial response, 13 had stable disease and 16 had progressive disease. No further evaluation or data analysis was made with respect to RR. Murata *et al*[22] evaluated RR in respect to hENT1 expression; radiographic RR was judged according to RECIST (Response Evaluation Criteria in Solid Tumors). Radiographic RR was not significantly correlated with hENT1 expression (*P* = 0.665).

DISCUSSION

Based on the data collected from the selected studies, there is evidence that hENT1 expression is a predictive marker for pancreatic cancer patients treated with gemcitabine. Patients with high expression of hENT1 had significantly longer OS in all included studies that evaluated this outcome measurement. These results are in accordance with other studies of gemcitabine outcome correlated with hENT1 expression in other types of tumors,

Table 3 hENT1 expression levels, cut-offs and grouping

Ref.	Method	Grading	Reference cells	Groups (*n*)
Spratlin *et al*[9]	IHC	0-2 based on relative intensities of staining. 0 = absence of staining 1 = intermediate staining 2 = most intense staining	Langerhans cells, lymphocytes.	Dichotomized: Low = 0 (12) High = 1 and 2 (9)
Giovannetti *et al*[10]	RT-PCR	Gene-expression ratio with GAPDH, expressed as tertiles GAPDH/target gene ratio		Gene expression tertiles: Low < 1.06 (27) Intermediate 1.06-1.38 (28) High ≥ 1.38 (26) Dichotomized: By medians Low < 1.23 (44) High ≥ 1.23 (37)
Farrell *et al*[12]	IHC	Based on relative intensities. High = strong reactivity in > 50% of neoplastic cells. No = no staining in > 50% Low = all cases between High and No.	Lymphocytes	Dichotomized: No (18)[1] *vs* Low/high (73)[1]
Maréchal *et al*[3]	IHC	0-3 based on staining intensities 0 = no staining 1 = weakly positive 2 = moderately positive 3 = strongly positive Final score calculated: multiplying intensity score and the percentage of the specimen. Weighted score 0-300	Langerhans cells Lymphocytes	Dichotomized: Low < 80 (26) (final score) High = ≥ 80 (19)
Fujita *et al*[8]	RT-PCR	Level of mRNA calculated from standard curve constructed with total RNA from Capan-1, a human pancreatic cancer cell line		mRNA split into high/low groups using recursive descent partitioning. Cut-off 0.5 Low (26)[1] High (14)
Maréchal *et al*[1]	IHC	0-2 based on staining intensities Quantified as Farrell	Lymphocytes	Dichotomized: Low/moderate (136)[1] High (86)[1]
Kawada *et al*[2]	IHC	0-2 based on staining intensities. 1 = same intensity as control.	Langerhans cells	Negative = 0-1 (41) Positive = 2 (22)
Morinaga *et al*[21]	IHC	Staining intensity and percentage of positive tumor cells scored and given a hENT1-score by calculating the two Staining 0-3 where 0 = no 1= weakly pos 2 = moderately pos 3 = strongly pos Percentage: 0 = no positive 1 ≤ 50% positive cells 2 = 50%-80% positive cells 3 = ≥ 80%		Low = hENT1 score 0-3 (11) High = hENT1 score 4-6 (16)
Murata *et al*[22]	IHC	Staining intensity + extent of positive staining Intensity: 0 = no staining 1 = weakly positive 2 = moderately positive 3 = strongly positive Extent staining: High = score 3 > 50% cells Low = score 0 or 1 > 50% Intermediate = all others	Langerhans cells	Dichotomized: Negative = low and intermediate (16) Positive = high (39)
Nakagawa *et al*[23]	IHC	Staining intensities: 0 = not stained 1 = faintly stained 2 = weakly stained 3 = as strongly as islet cells	Langerhans cells	Low = grade 0 or 1 in > 50% (31) High = grade 2 or 3 in > 50% of cells (78)

[1]In gem arm. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

including biliary tract cancer[24], cholangiocarcinoma[25], bladder cancer[26] and non-small cell lung cancer[27].

Since there is no standardized protocol for the grading of hENT1 expression, the methods used differed between the included studies (Table 3). The majority used immunohistochemistry to evaluate hENT1 expression

Table 4 Results

Ref.	Median survival all patients (95%CI)	OS Univariate analysis median (95%CI) or HR (95%CI) *P*-value	OS Multivariate analysis HR (95%CI)	DFS Univariate analysis median (95%CI) or HR (95%CI) *P*-value	DFS Multivariate analysis HR (95%CI)	Main conclusions
Spratlin *et al*[9]	11.0[1] (6.8-17.5) 5.0[1] (2.8-12.2)	(mo): High = 13 (4.2-20.4) Low = 4 (1.5-6.9) *P* = 0.01		NR		Pat with detectable hENT1 had sig longer OS compared with pat with low hENT1
Giovannetti *et al*[10]	13.3 (10.9-15.7)	(mo): Low = 8.48 (7.01-9.95) Inter = 15.74 (13.84-17.63) High = 25.69 (17.64-33.74) *P* ≤ 0.001 2 groups: Low = 12.42 (8.18-16.66) High = 22.34 (16.34-28.34) *P* ≤ 0.001	Low = 5.34 (2.28-12.50) Inter = 1.07 (0.46-2.49) High = 1 *P* < 0.0001 2 groups: HR = 4.21 *P* ≤ 0.001	Palliative (mo): Low = 5.85 (2.75-8.95) Inter = 10.09 (9.63-10.54) High = 12.68 (2.89-22.47) *P* = 0.02 Adjuvant (mo): Low = 9.26 (3.86-14.67) Inter = 12.91 (9.31-16.51) High = 20.43 (13.27-27.60) *P* ≤ 0.01		hENT1 expression was significantly correlated with outcome - pat with high hENT1 had longer OS
Farrell *et al*[12]	NR	(HR): Low/High = 0.51 (0.29-0.91) No = 1 *P* = 0.02	Low/high = 0.40 (0.22-0.75) No = 1 *P* = 0.03	(HR): Low/High = 0.57 (0.32-1.001) No = 1 *P* = 0.05	Low/high = 0.39 (0.21-0.73) No = 1 *P* = 0.003	hENT1 expression was ass with longer OS, DFS in pat receiving gem. hENT1 is a relevant predictive marker for gem outcome
Maréchal *et al*[3]	21.9 (3.3-107.4)	(HR): High = 1 Low = 3.88 (1.78-8.92) *P* = 0.0007	High = 1 Low = 3.42 (1.44-8.81) *P* = 0.0005	(HR): High = 1 Low = 3.55 (1.65-7.63) *P* = 0.02	High = 1 Low = 3.17 (1.43-6.73) *P* = 0.0004	Pat with high hENT1 had sig longer OS and DFS compared to low hENT1
Fujita *et al*[8]	NR	(mo): High = 45 Low = 16.5 *P* = 0.011	(RR): Low = 2.980 (0.964-10.86) *P* = 0.2 (not sig)	(mo): High = 25 Low = 8 *P* = 0.11 (not sig)	NR	Low hENT1 ass with shorter OS in gem-group
Maréchal *et al*[1]	32.0 (26.4-34.3) (GEM-group)	(HR): High = 0.43 (0.29-0.63) Low/Mod = 1 *P* < 0.0001	*n* = 222[2] High = 0.34 (0.22-0.53) Low/Mod = 1 *P* < 0.0001	NR	NR	High hENT1 predicts longer OS in pat treated with adj gem. Absence of gem - hENT1 lacks prognostic value
Kawada *et al*[2]	NR	Positive *vs* negative *P* = 0.352	Positive/negative *P* = 0.503	NR	NR	DSS tended to be better in the hENT1-neg group but not statistically sig
Morinaga *et al*[21]	NR	(mo): Low = 11.8 (6.9-16.6) High = 22.2 (11.5-32.9) *P* = 0.024 (HR): Low = 1 High = 0.366 (0.148-0.906) *P* = 0.030	Low = 1 High = 0.327 (0.128-0.835) *P* = 0.019	(mo): Low = 7.3 (3.6-11.1) High = 9.3 (4.2-14.5) *P* = 0.022 (HR): Low = 1 High = 0.362 (0.146-0.898) *P* = 0.028	Low = 1 High = 0.558 (0.214-1.452) *P* = 0.232	High hENT1 sig ass with longer OS in pat receiving adj gem after resection
Murata *et al*[22]	24.3	(HR): Positive = 1 Negative = 3.04 (1.45-6.37) *P* = 0.0037	Positive = 1 Negative = 3.15 (1.35-7.37) *P* = 0.008	(HR): Positive = 1 Negative = 2.34 (1.22-4-47) *P* = 0.011	Positive = 1 Negative = 1.76 (0.85-3.66) *P* = 0.129	Sig longer OS, RFS in pat with pos hENT1
Nakagawa *et al*[23]	OS: 34.9 DFS: 17.8	(5y-SR %): High = 38 Low = 13 *P* = 0.001	High = 1 Low = 3.16 (1.65-6.06) *P* = 0.001	(5y-SR %): High = 30 Low = 17 *P* = 0.004	High = 1 Low = 2.70 (1.52-4.83) *P* = 0.001	hEN1 expression is predictive of the efficacy of adj gem-based chemotherapy after resection

[1]From diagnosis/from treatment; [2]*n* = 222 in multivariate analysis. Ass: Associated; pat: Patient; sig: Significant; adj: pos: Positive; op: Operation; HR: Hazard ratio; SR: Survival rate; RFS: Recurrence-free survival.

in the tumor cells. This is the main method for assessing biomarkers in histopathology. Most studies in this review using this method of evaluation had 2 independent assessors (blinded to each other and to patient outcomes) to make the grading for higher quality and better precision. The different ways of grading protein expression is an issue that needs to be considered when assessing the results of independent studies as well as the results of this review. There is a need for standardized protocols to achieve better homogeneity across studies when it comes to grading the protein expression of hENT1 in pancreatic tumor cells.

Gemcitabine is the standard treatment for patients with pancreatic cancer. This is based on several studies where gemcitabine has shown a survival benefit compared with other treatment regimes[1,28-30]. In this review, the treatment differed amongst the included studies, but they all used gemcitabine as the basis for chemotherapy. Most studies were performed in resectable patients, but the predictive value of hENT1 was also confirmed in unresectable patients[9,10]. Kawada *et al*[2] used neoadjuvant chemoradiation and their results showed, in contrast with all the others, a trend towards better DSS in patients with low expression of hENT1, although the results were not statistically significant. They did use a slightly different outcome measurement that may have influenced the results. Even though the result was not statistically significant it raises some questions that are important in the discussion about the different treatment regimens across the studies. In the case of neoadjuvant treatment or neoadjuvant chemoradiation, tumor cells with high expression of hENT1 may be destroyed before the tumor samples are collected and will therefore give misleading information. This creates interesting issues as to when and how the tumor cells should be analyzed. Fine needle aspiration is discussed as an option for retrieving tumor cells for evaluation of hENT1. This method can be used before resection and may therefore be an effective tool to identify which patients may benefit from neoadjuvant treatment with gemcitabine. Future studies are needed in this area.

One study[12] was a *post hoc* analysis of a randomized controlled trial, which is of course rated higher methodologically than are retrospective cohort studies. The common opinion is that a systematic review exhibits the greatest strength if the majority of the included studies are randomized controlled trials or at least prospective trials. However, no such trials have been made within this area, but the need for a review was still considered to be necessary. The retrospective design of the included studies implies that we need to consider reporting and selection bias when analyzing the results.

REMARK[14], which is a relatively new assessment tool, was used for quality evaluation in this review. The maximum score in REMARK is 20, and the average score in the included articles was 12.6 within the range of 9-17. Since this is a relatively new tool, there is not much information as to what quality is considered high, and what is low. In this review, the included articles were of relatively similar quality (Table 2) according to REMARK.

The results of this review are important to the consideration of future treatment options for patients with pancreatic cancer. According to this review, hENT1 has been proven to be a predictive marker for gemcitabine outcome, thus there are a few considerations to be made. First, if we can alter the expression of hENT1 in the tumor cells to create a higher expression, more patients would benefit from gemcitabine treatment and survive longer. Pretreatment with thymidylate synthase inhibitors has proven to increase the expression of hENT1 in tumor cells *in vitro*[6]. This might be a way to alter the expression *in vivo* as well, but further studies are needed. The second option is to find another way for gemcitabine to enter the tumor cells and exert its toxic effect. Research in this area is currently under way, and progress should enable more personalized treatment options for pancreatic cancer patients.

Another aspect for the future is the cost of overtreatment with gemcitabine in patients who do not benefit from it. According to one study conducted on a Swedish pancreatic cancer cohort, EUR 8.6 million would be saved each year in Sweden if hENT1 testing were used to select patients for gemcitabine therapy[31].

CONCLUSION

This review provides evidence that hENT1 is a predictive marker for pancreatic cancer patients treated with gemcitabine. However, standardized procedures for evaluating and grading hENT1 expression need to be established. Additionally, more research and preferably prospective trials or randomized controlled trials in this area are needed to confirm the results of this review.

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2014 ADVANCES IN PANCREATIC CANCER

Managing malignant biliary obstruction in pancreas cancer: Choosing the appropriate strategy

Brian R Boulay, Mayur Parepally

Brian R Boulay, Mayur Parepally, Division of Gastroenterology and Hepatology, University of Illinois Hospital and Health Sciences System, Chicago, IL 60612, United States
Author contributions: Boulay BR and Parepally M performed literature search and contributed equally to the creation of this topic highlight.
Correspondence to: Brian R Boulay, MD, MPH, Division of Gastroenterology and Hepatology, University of Illinois Hospital and Health Sciences System, 840 S Wood Street, MC 716, Chicago, IL 60612, United States. bboulay24@gmail.com
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Abstract

Most patients with pancreatic cancer develop malignant biliary obstruction. Treatment of obstruction is generally indicated to relieve symptoms and improve morbidity and mortality. First-line therapy consists of endoscopic biliary stent placement. Recent data comparing plastic stents to self-expanding metallic stents (SEMS) has shown improved patency with SEMS. The decision of whether to treat obstruction and the means for doing so depends on the clinical scenario. For patients with resectable disease, preoperative biliary decompression is only indicated when surgery will be delayed or complications of jaundice exist. For patients with locally advanced disease, self-expanding metal stents are superior to plastic stents for long-term patency. For patients with advanced disease, the choice of metallic or plastic stent depends on life expectancy. When endoscopic stent placement fails, percutaneous or surgical treatments are appropriate. Endoscopic therapy or surgical approach can be used to treat concomitant duodenal and biliary obstruction.

Key words: Pancreatic neoplasms; Cholestasis; Extrahe-

patic; Stents; Biliary tract diseases

Core tip: Biliary obstruction is a common problem in pancreatic malignancy. Relief of obstruction is commonly performed using endoscopic stent placement. Clinical setting determines the strategy, including whether decompression is needed and which stent type is most appropriate. Self-expanding metallic stents have longer patency than plastic stents and are preferred in most settings. When endoscopic therapy fails, percutaneous or surgical strategies may be used.

INTRODUCTION

Malignant bile duct obstruction can be a devastating consequence of pancreatic cancer. Its development may contribute to poor outcomes including cholangitis, delay in treatment (including chemotherapy or surgery), decreased quality of life and increased mortality. Pancreatic ductal adenocarcinoma has a dismal five-year survival rate of only 6%, and biliary obstruction correlates with decreased survival times[1]. As many as 70% of patients have some degree of biliary obstruction at the time of their initial diagnosis with pancreatic cancer[2]. In most of these situations, the adverse nature of biliary obstruction can be improved with decompression. For purposes of palliation, decompression can improve patient comfort by relieving jaundice and pruritus[3]. It can also facilitate treatment by allowing total bilirubin levels to drop to less than 1.5 times the upper limit of normal, which is necessary to prevent toxicity in some chemotherapy regimens[4].

The role of biliary stents in achieving biliary decompression has been well established for the past 20 years, with more recent studies showing an increased role for self-expandable metal stents (SEMSs) compared to plastic stents[5-10]. Endoscopic biliary stenting is technically successful in over 90% of attempted cases[9]. Thus, endoscopic retrograde cholangiopancreaticogram (ERCP) with biliary stent placement has become the standard of care in situations where biliary decompression is desired.

The purpose of this article is to review the efficacy and outcomes of different strategies including stenting in relieving malignant bile duct obstruction. The strategy and choice of stent may differ based on clinical scenario and disease stage, including resectable disease, locally advanced disease treated with neoadjuvant therapy, and metastatic disease in which only palliative therapy is available. Second-line methods including percutaneous drainage, endoscopic ultrasound (EUS) guided biliary drainage and surgical bypass will be discussed and compared to current methods utilized as the standard of care. In cases of concurrent gastric outlet obstruction and malignant biliary obstruction, strategies such as double stenting, double surgical bypass, and EUS guided biliary drainage with duodenal stenting will also be discussed.

BACKGROUND ON STENTS

Polyethylene or plastic stents are used for relief of biliary obstruction in numerous settings, and offer excellent patency for short-term use. These stents are available in multiple diameters ranging from 7 French to 11.5 French, though 10 French stents are the most commonly used with distal common bile duct obstruction. The benefits of polyethylene stents include low cost for the prosthesis itself, as well as removability at the time of surgical procedures.

One of the factors that initially led to plastic stents being used preferentially in pancreatic cancer was the notion that uncovered SEMS could complicate pancreaticoduodenectomy by interfering with transection of the bile duct proximal to the resection specimen[6]. Experience has shown that as long as > 2 cm of common hepatic duct is exposed proximal to the SEMS, then surgery is no more complex than in the presence of a plastic stent[7]. Thus, the choice of stents in treatment of malignant biliary obstruction relies on other factors such as cost-effectiveness, expected length of survival, and certainty of the diagnosis of malignancy.

Any stent is subject to occlusion in the setting of distal common bile duct obstruction, though the mechanisms can differ by stent design. For plastic stents, the development of biofilm and bacterial colonization is the most important factor[8]. For uncovered SEMS, tissue ingrowth through the mesh interstices at the level of the tumor remains the most likely source of occlusion. For partially or fully covered SEMS, occlusion may occur due to stent migration, overgrowth of tissue at the ends of the stent, or food debris. Duodenal contents can also flow back up the biliary system in a retrograde fashion, as demonstrated by contrast studies[11]. This duodenal biliary reflux may cause stent occlusion in any type of stent.

RESECTABLE DISEASE

The choice of modality for decompression varies by the clinical setting and expected treatment for each patient. For pancreatic cancer without local advancement or metastases, prompt surgical resection is the definitive method of treatment and the only hope of cure[12]. There is debate over whether jaundice should be relieved prior to surgery. In theory, relieving jaundice would improve surgical outcomes by overcoming the impaired immune response and coagulopathy associated with cholestasis. Over the past 10 years, multiple studies have examined the role of biliary decompression with stenting in localized disease prior to pancreaticoduodenectomy. In many cases, preoperative biliary drainage was found to be associated with increased complications including infections, abscesses, pancreatic fistulas and wound infection[13,14]. Authors have hypothesized that these complications are due to infection of bile from instrumentation with foreign objects into the biliary system[13,15].

In 2010, a Dutch multicenter randomized trial demonstrated that preoperative biliary drainage and stenting was associated with increased complications compared to surgery alone in resectable disease[16]. In this trial, 202 patients were randomized to undergo either preoperative biliary drainage followed by surgery within 4-6 wk, or surgery alone within 1 wk of diagnosis. Rates of serious complications were 39% in the early - surgery group and 74% in the group with biliary drainage (RR = 0.54, $P < 0.001$). There was also no mortality benefit or shortened length of stay with preoperative drainage.

The trial garnered much attention due to its large size, as well as its randomized prospective multicenter design. However, some methodological issues may limit the generalizability of the above conclusions. As the preoperative biliary drainage group waited 4-6 wk for surgery, one could theorize that a shorter interval between stent placement and surgery may have allowed fewer stent-related complications. (The authors chose this length of time prior to surgery to allow normal synthetic and clearance functions of the liver). The use of plastic stents rather than larger diameter SEMS was also cited as a factor in the poor performance of the biliary decompression group. These issues may warrant further studies to address them and may guide clinical practice.

Based on the data from this study, routine preoperative biliary decompression is not currently recommended. However, for patients who present with cholangitis or intractable pruritus stent placement is an appropriate intervention prior to pancreaticoduodenectomy.

LOCALLY ADVANCED DISEASE AND NEOADJUVANT THERAPY

Plastic stents vs self-expanding metal stents

The poor long-term survival for pancreatic cancer even in surgically resected disease has prompted interest in the use of neoadjuvant therapy in operable pancreatic cancer to improve patient survival[17]. This strategy has also been used in locally advanced cases that may require downstaging to permit eventual surgical resection. Patients who receive neoadjuvant therapy such as gemcitabine-based regimens require biliary decompression to allow the safe use of such chemotherapeutic agents[4]. Biliary stenting during the neoadjuvant period has been the common method for achieving this decompression, with the goal of stent patency up until surgery. Unfortunately, the performance of plastic stents during the preoperative period has been lackluster. A retrospective study showed that the median time from stent placement to surgery was 150 d, while the median duration of stent patency was 134.5 d[5]. In order to achieve a longer duration of stent patency during this time, SEMSs have been studies for use rather than plastic stents.

Multiple studies have now demonstrated that the use of SEMSs in such patients lead to improved outcomes during neoadjuvant therapy[5,6,10,11,18]. A retrospective review of plastic stent performance during neoadjuvant therapy revealed that over half the patients with plastic stents required repeat stent exchange due to stent occlusion or cholangitis[5]. Adams *et al*[10] demonstrated in 2012 that in a retrospective cohort of 52 patients, the complication rate was almost 7 times higher with plastic stents. It was also estimated the rate of hospitalization for these cases was 3 times higher than patients with metal stents. Although SEMSs are more expensive than plastic stents, data thus far indicates that their superiority in patency and improved patient outcomes make them the safer and ultimately the more cost effective choice for patients in whom attempted surgical resection is planned[19].

TYPES OF SEMSs: NO PERFECT CHOICE

More recent literature has focused on comparing the different types of SEMS for use in patients undergoing neoadjuvant therapy or in palliative cases. Major categories of SEMSs include uncovered (USEMSs) and covered (CSEMSs) groups. USEMSs have a mesh design that allows them to embed in the biliary duct wall but it also makes them susceptible to tissue ingrowth, which can lead to occlusion in as many as 20% of patients. CSEMSs were designed to prevent tissue ingrowth, but because of this they are known to have increased rates of migration[20].

Several studies have demonstrated the trade-off between tissue ingrowth in USEMS and migration in CSEMS. A meta-analysis by Saleem *et al*[21] concluded that CSEMSs had a significantly longer duration of patency than USEMSs (average of 61 more days) in palliative cases, but also noted their increased incidence in migra-

tion (RR = 8.11). The study also noted that CSEMSs and USEMSs had similar rates of cholecystitis (approximately 2% in each group). A more recent retrospective cohort study by Lee *et al*[22] had different conclusions, with no difference in overall recurrent obstruction (CSEMSs 35% *vs* USEMSs 38%) among 749 patients. While tumor ingrowth associated with obstruction was higher in the USEMS group (76% *vs* 9%, $P < 0.001$), other mechanisms of obstruction still occurred with CSEMSs, including tumor overgrowth, sludge formation, food debris, and migration. CSEMSs were found to have higher rates of migration (36% *vs* 2%, $P < 0.001$) and acute pancreatitis (6% *vs* 1%, $P < 0.001$). Despite its large size, limitations to the study include its nonrandomized and retrospective design, with lack of uniform follow-up data for patients.

In an effort to decrease rates of migration while maintaining the patency achieved with CSEMSs, partially covered SEMSs have also been used in practice. Through subgroup analysis, Saleem *et al*[21] did not find any difference in rate of migration or stent patency in partially covered SEMSs compared to fully covered SEMSs. A multicenter randomized trial by Telford *et al*[23] compared partially covered SEMSs to USEMSs, and found no significant difference in rates of obstruction and patient survival. It did note a statistically significant increase in adverse events (62% *vs* 44%, $P = 0.046$) and stent migration (12% *vs* 0%, $P = 0.0061$) with partially covered SEMSs. Limitations to this study included some imbalance in the distribution of patients to the treatment group, and difficulties in recruiting an adequate number of patients.

A more recent study by Kitano *et al*[24] demonstrated the use of a modified CSEMS aimed at reducing stent migration. The anti-migration system in this model of CSEMS used low axial force and uncovered flare ends, and was compared to USEMSs of a similar design. A total of 120 patients were included in the prospective randomized multicenter study. Patients were randomized to receive the modified CSEMS or USEMS. The CSEMS cohort had significantly longer durations of stent patency (mean of 219.3 d *vs* 166.9 d, $P = 0.047$) and less need for reintervention (23% *vs* 37%, $P = 0.08$) compared to USEMSs. The rate of tumor ingrowth was also significantly less in the CSEMSs group (0% *vs* 25%, $P < 0.01$). Neither group demonstrated stent migration; survival time (median 285 d in CSEMSs *vs* 223 d in USEMSs, $P = 0.68$) and serious adverse events also did not differ significantly. It would be useful for a study to compare these CSEMSs with partially covered SEMSs and USEMSs concurrently to show which type has overall superiority.

The limitations found in each category of SEMSs make it difficult to use one type as the ideal stent for biliary decompression in obstruction caused by pancreatic cancer. In addition, some of the studies do have conflicting data, which indicates the need for further investigation to determine which type of stent should be used in the future. While SEMS show improved patency rates compared to plastic stents, occlusion still occurs with disturbing frequency. A recent Korean review of SEMS

patency among 107 patients with unresectable pancreatic cancer showed stent occlusion in 36% of patients during a median survival period of 33 d[25]. This appears consistent with the experience of other large studies as detailed above. There is still a need for other types of SEMSs that would minimize the flaws of existing designs.

ALTERNATIVE STENT DESIGNS AND STRATEGIES

Drug eluting stents have been designed in an attempt to improve SEMS patency by eluting a chemotherapeutic agent such as paclitaxel to prevent tumor ingrowth and stent occlusion[26]. Prior studies have shown that they effectively inhibit cells responsible for stent occlusion[27] and can be safely used in animal models[28] and humans[29]. Recently Jang *et al*[30] conducted a multicenter prospective comparative study to compare the efficacy of this type of stent to covered SEMS in patients with unresectable distal malignant biliary obstruction. In a non-randomized fashion, 60 patients were enrolled into a paclitaxel coated SEMS group while 46 were enrolled to the covered SEMS group. There was no significant difference in rates of stent patency between both groups. There are ongoing efforts to design new drug eluting stents with different chemotherapeutic agents such as gemcitabine. Further trials are needed to determine whether these stents can improve upon the performance of the current generation of SEMSs.

Other stents have been designed to prevent reflux of duodenal contents into the biliary system, which is another known cause of stent occlusion[11]. Dua *et al*[31] demonstrated through a randomized prospective trial that an anti-reflux plastic biliary stent (AR-PBS) could stay patent for a longer duration compared to traditional plastic stents (median patency 145 d for AR-PBS compared to 101 d for PBS, $P = 0.002$). Subsequent studies focused on the use of anti-reflux metal stents (ARMSs). In a retrospective single center case series, Hu *et al*[32] described the use and outcomes of ARMSs in 23 patients. Median patency of the stents was 14 mo and overall patency was reported at 3, 6 and 12 mo (95%, 74%, 56% respectively). A separate study group designed their own anti-reflux SEMS (AR-SEMS), and in their own prospective case series described the placement of AR-SEMSs in five patients with unresectable hilar malignant biliary obstruction[33]. Stent occlusion occurred early in four patients with patency durations ranging 4-26 d, and the fifth patient's stent remained patent for 235 d. The authors noted that the outcomes may have been different from Hu *et al*[32] due to differences in stent design, as their stent was an end-flared type. Hu *et al*[32], in comparison, used stents that were hemispheric type and were covered in a hemispheric silicon membrane. Further studies need to be conducted to confirm the efficacy of AR-SEMSs and show superiority to current stents before they can be applied to more widespread use.

RECOMMENDED STRATEGY FOR STENTING IN LOCALLY ADVANCED DISEASE

The use of a plastic stent for biliary decompression in locally advanced disease appears largely unwarranted based on the studies reviewed above. However, practitioners may be hesitant to place a SEMS if the diagnosis of malignancy is uncertain at that time of ERCP. Routine use of EUS-guided fine needle aspiration with on-site cytologic review is limited to certain centers, and the differential diagnosis for distal common bile duct strictures may include chronic pancreatitis or autoimmune cholangiopathy, in which case a removable stent is the best option. With the recent availability of fully covered SEMS and data to show their comparable efficacy with uncovered SEMS, the use of a fully covered SEMS appears to be a sound strategy when suspicion of malignancy is high and life expectancy is greater than 4 mo (Figure 1).

Palliative stenting

Another area of focus in malignant biliary obstruction is the placement of stents for palliative purposes in incurable pancreatic cancer. The rationale for their placement is similar in many respects to that of patients undergoing neoadjuvant therapy: relief of jaundice and pruritus, normalization of bilirubin levels to allow palliative chemotherapy, and prevention of other adverse outcomes such as cholangitis and frequent hospitalizations. However in patients with advanced disease and shorter life expectancies, it may be difficult in justifying the use of SEMSs as long- term patency is less of a goal in these cases. A meta-analysis by Moss *et al*[19] showed that SEMSs cost 15-40 times more than most plastic stents, and are only cost effective if the patient survives > 4 mo. One of the included studies demonstrated that the presence of liver metastases was independently related to survival ($P < 0.0005$ with multivariate analysis), with a median survival of 2.7 mo compared to 5.3 mo without liver metastases[34]. Cost analysis demonstrated that plastic stents were more cost effective in the patients with liver metastases compared to SEMSs. Soderlund *et al*[35] reached similar conclusions, showing that the survival period for patients with distant metastases was similar to patency time in plastic stents. They concluded that SEMSs should only be reserved for patients who did not have distant metastases. While some other prognostic factors have been identified to predict mortality in pancreatic cancer, the data is limited and generally used to determine surgical risk[36-38].

Recently there has been growing use of double layer stents (DLSs) as a cost-effective alternative to SEMSs in palliative cases. Such stents are designed with a stiff outer layer to allow stricture cannulation and a smooth inner layer that is less likely to occlude. A recent retrospective review demonstrated DLSs had a longer duration of stent patency than plastic stents (95 d *vs* 59 d, $P = 0.014$). There was also no significant difference in patency be-
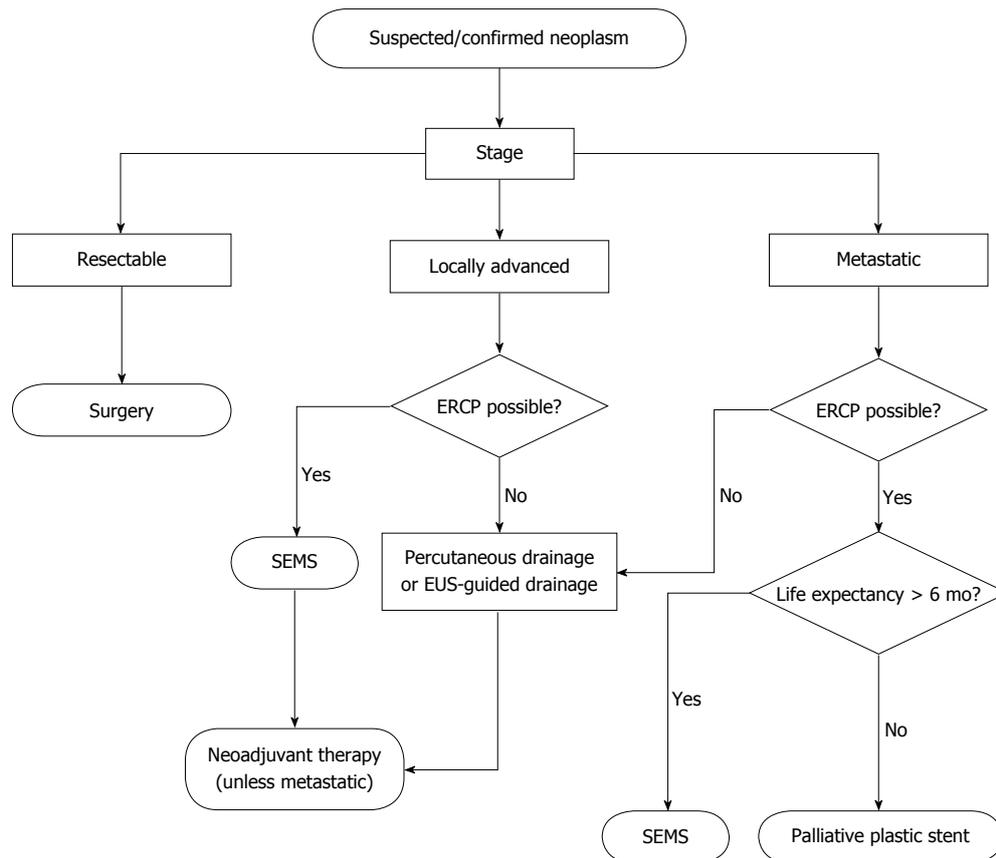
Figure 1 Algorithm for relief of malignant biliary obstruction from pancreas cancer. SEMS: Self-expanding metallic stents; ERCP: Endoscopic retrograde cholangiopancreaticogram; EUS: Endoscopic ultrasound.

tween the DLS and SEMS group[39]. These results were consistent to past studies[40-42], and may pave the way in routine use of DLSs in palliative relief of obstruction due to advanced pancreatic cancer.

OTHER METHODS FOR BILIARY DRAINAGE

Percutaneous transhepatic biliary drainage

In cases where patients are not candidates for ERCP or have failed attempted transpapillary stent placement, percutaneous transhepatic cholangiography (PTC) has traditionally been used as a method for biliary drainage. This method can offer the same benefits of biliary decompression in improving patient comfort and preventing adverse outcomes. In most cases an internal-external biliary drain is passed through the site of malignant biliary obstruction to the duodenum, where it can reestablish internal bile drainage and normal enterohepatic circulation. Efforts are made to discontinue the external drainage component unless it continues to have high output or the patient is in a state of sepsis, in which case internalization is delayed. In some of these situations, or cases when exclusively external percutaneous biliary drains are placed due to inability to transverse the site of obstruction, having continued external drainage can be cumbersome and

uncomfortable for patients. External drains can require significant maintenance, including emptying and flushing of the drain as well as routine drain exchange to prevent occlusion[43]. It should also be noted that PTC can cause bacteremia, cholangitis and hemobilia. Internal-external and external biliary drains can also be prone to leakage, dislodgement and obstruction. However, in cases when ERCP fails these drains are an appropriate means of biliary decompression.

Percutaneous stent placement

Percutaneous stent placement has been another option to relieve malignant biliary obstruction, but historically have been avoided for similar reasons as above. In a study conducted 25 years ago, Speer *et al*[44] randomized patients into groups receiving biliary stents by percutaneous or endoscopic routes. It concluded that endoscopically placed stents had improved efficacy and lower mortality, due to complications including hemorrhage and bile leaks in the percutaneous group. These results have contributed to the use of endoscopic stent placement as first-line treatment. However, recent studies using improved technology including the percutaneous placement of SEMSs have shown to be safe and effective[45-47]. This may lead to more routine us of percutaneous stent placement, especially in cases when ERCP is not possible.

EUS guided biliary drainage

Other endoscopic alternatives are being used in relieving malignant biliary obstruction not amenable to stent placement *via* ERCP. Endoscopic ultrasound-guided biliary drainage (EUS-BD) has been demonstrated to be a safe and effective means of biliary drainage. EUS-BD can be achieved by multiple techniques including EUS-guided rendezvous, EUS-guided choledochoduodenostomy (EUS-CDS), and EUS- guided hepatic gastrostomy (EUS-HGS)[48]. In situations when the endoscope can reach the ampulla, rendezvous can be achieved by inserting an FNA needle into the common bile duct or left intrahepatic duct under EUS guidance, followed by navigating a guidewire through the bile duct past the stricture into the duodenum. A duodenoscope can then be used to allow over-the-wire cannulation with ERCP and retrograde stent placement. When the papilla cannot be reached due to malignant obstruction of the duodenum, EUS-CDS and EUS- HGS offer ways to create tracts to the bile duct with extrahepatic and intrahepatic approaches respectively. Both these methods also utilize guidewires that are advanced past the ampulla into the duodenum, which is followed by anterograde dilation of the tract and stent placement. At this time, these procedures remain technically complex and limited to high-volume centers with expertise in therapeutic endoscopy[49]. Complications may include bile leak, bleeding, or pneumoperitoneum. Future trials will further assess the efficacy of these methods and seek to improve their feasibility and safety.

Adjunctive techniques may hold some promise for improving the long-term performance of stents in malignant obstruction. An emerging method known as endobiliary bipolar radiofrequency ablation (RFA) has been able to achieve safe and effective biliary decompression and patency. The procedure involves placement of a bipolar RFA catheter across the biliary stricture under fluoroscopic guidance, with subsequent energy delivery to cause local tumor necrosis. In an open-label pilot study, Steel *et al*[50] recruited 22 patients with unresectable obstruction with the goal of using bipolar RFA prior to placing uncovered SEMSs in each patient. In all, 21 of the patients had successful RFA catheter deployment and SEMS placement. At 90 d, 76% (16/21) of those patients still had stent patency. Further studies are investigating this novel method, and will need to determine the long-term efficacy and safety of RFA, along with direct comparison to existing SEMS performance.

Surgical biliary drainage

Finally, surgical biliary bypass for drainage remains an option. Glazer *et al*[51] performed a meta-analysis of trials comparing surgical bypass and endoscopic stent placement in patients with unresectable pancreatic cancer. Recurrent biliary obstruction was less likely in surgically treated patients (3.1% *vs* 28.7% of stent-treated patients) over a mean survival time of 4 mo. The length of hospital stay for either treatment type was fairly long (21.8 d for surgical bypass, 14.6 d for stent treatment) and the major-

ity of the studies used older data which preceded the use of SEMS. Another meta-analysis using the same studies demonstrated high rate of complications in surgical bypass, but similar overall mortality to patients treated with endoscopically placed stents[52]. Both of these meta-analyses included studies in which plastic biliary stents were the stent of choice; newer data comparing surgical bypass to SEMS is lacking but might be expected to yield a different result favoring endoscopic treatment. Nonetheless, surgical biliary bypass remains an appropriate treatment for patients with a life expectancy exceeding six months.

MANAGEMENT OF CONCURRENT GASTRIC OUTLET AND BILIARY OBSTRUCTION

Surgical bypass can also be used to relieve concomitant malignant biliary and gastric outlet obstruction caused by pancreatic cancer. However many patients with this presentation are either poor surgical candidates, or may decline a comparatively invasive surgery that would include both gastrojejunostomy and biliary bypass. With the advent of enteral and biliary SEMSs in the past decade, biliary and gastroduodenal obstruction can be relieved using endoscopic double stenting in a safe and less invasive manner. In 2002, Kaw *et al*[53] conducted a retrospective review of 18 patients who underwent simultaneous biliary and duodenal SEMS placement. Median survival time for the cohort was 78 d and only 4 out of 18 patients experienced recurrent obstruction (2 with biliary obstruction and 2 with duodenal obstruction; all were successfully stented). Similar results have been obtained in subsequent studies[54-56], with SEMS placement at the duodenal stricture followed by an endoscopic approach to the papilla for ERCP guided biliary stent placement. If the stricture involved the papilla, biliary stenting was performed through the mesh of the duodenal SEMS with high rate of success. In patients where the transpapillary approach fails, EUS-BD can be performed after the initial endoscopic duodenal SEMS placement to allow biliary stenting[55,56]. Given the limited use of EUS-BD and its recent development, this approach may be limited to high volume centers.

CONCLUSION

Malignant biliary obstruction caused by pancreatic cancer is associated with poor outcomes and decreased survival in patients. Biliary decompression through the interventions discussed in this review can be performed to improve patient quality of life and mortality. Although the available data seems to indicate that resectable disease should proceed straight to surgery, there may still be benefit in preoperative stenting if surgery will be delayed or if jaundice is associated with complications. The role for stenting in biliary decompression is clearer in locally advanced disease and with incurable patients for palliative

purposes.

Numerous studies have shown the overall superiority of SEMS compared to plastic stents in terms of long-term stent patency and improved patient outcomes. At this time, there is no ideal type of SEMS as both uncovered and covered SEMSs are associated with their own benefits and limitations. A recent study has demonstrated a modified covered SEMS with less migration and similar patency to traditional CSEMS, but further studies using this stent will be needed to demonstrate clear superiority. Drug eluting stents may offer a more effective option in the future, although current designs have not shown superiority to the current generation of SEMSs. In palliative stenting, it is potentially not cost effective to use SEMSs in patients with shorter life expectancy given their expense relative to plastic stents. The presence or absence of distant metastases can help guide what type of stent should be used, but predictive mortality models may offer a way to further stratify patients in an accurate and cost effective fashion. Double layer stents may provide a less expensive option for some patients, while still maintaining superiority to regular plastic stents.

Although percutaneous transhepatic cholangiography with internal-external and external drainage catheters have been used when ERCP and stent placement is not possible, it has some disadvantages such as patient discomfort, need for maintenance and routine exchanges, and can be associated with hemobilia, cholangitis and sepsis. Percutaneous SEMS placement may be an underutilized strategy given successful trials utilizing it, but it can still have some of the complications mentioned above. EUS-guided biliary drainage and RFA ablation may provide a better alternative, but require more research into their safety and efficacy. Surgical bypass may be appropriate in cases when life expectancy exceeds 6 months, or in patients with concomitant duodenal obstruction. Endoscopic double SEMS placement or EUS-BD with endoscopic duodenal stent placement may be safe and less invasive methods for palliation of malignant and duodenal obstruction due to pancreatic cancer.

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WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Pancreatic cancer: Advances in treatment

Somala Mohammed, George Van Buren II, William E Fisher

Somala Mohammed, George Van Buren II, William E Fisher, The Elkins Pancreas Center, Michael E DeBakey Department of Surgery, and Dan L Duncan Cancer Center, Baylor College of Medicine, Houston, TX 77030, United States
Author contributions: Mohammed S, Van Buren II G, Fisher WE contributed to concept and design, drafting of article, critical revision and gave final approval of this paper.
Correspondence to: William E Fisher, MD, Director, Professor, The Elkins Pancreas Center, Michael E DeBakey Department of Surgery, and Dan L Duncan Cancer Center, Baylor College of Medicine, 6620 Main St, Suite 1450, Houston, TX 77030, United States. wfisher@bcm.edu
Telephone: +1-832-3551490 Fax: +1-713-6102489
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Abstract

Pancreatic cancer is a leading cause of cancer mortality and the incidence of this disease is expected to continue increasing. While patients with pancreatic cancer have traditionally faced a dismal prognosis, over the past several years various advances in diagnosis and treatment have begun to positively impact this disease. Identification of effective combinations of existing chemotherapeutic agents, such as the FOLFIRINOX and the gemcitabine + nab-paclitaxel regimen, has improved survival for selected patients although concerns regarding their toxicity profiles remain. A better understanding of pancreatic carcinogenesis has identified several pre-malignant precursor lesions, such as pancreatic intraepithelial neoplasias, intraductal papillary mucinous neoplasms, and cystic neoplasms. Imaging technology has also evolved dramatically so as to allow early detection of these lesions and thereby facilitate earlier management. Surgery remains a cornerstone of treatment for patients with resectable pancreatic tumors, and advances in surgical technique have allowed patients to undergo resection with decreasing perioperative morbidity and mortality. Surgery has also become feasible in selected patients with borderline resectable tumors as a result of neoadjuvant therapy. Furthermore, pancreatectomy involving vascular reconstruction and pancreatectomy with minimally invasive techniques have demonstrated safety without significantly compromising oncologic outcomes. Lastly, a deeper understanding of molecular aberrations contributing to the development of pancreatic cancer shows promise for future development of more targeted and safe therapeutic agents.

Core tip: Pancreatic cancer is a leading cause of cancer mortality. However, recent advances have improved our ability to treat patients with this highly lethal disease. This review article discusses some of the salient advances in the field, such as improvements in chemotherapeutic regimens, imaging technology, surgical technique, and our understanding of the pathogenesis of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the tenth most commonly diagnosed cancer and the fourth leading cause of cancer mortality in the United States; the overall 5-year survival is only 5%[1]. Even patients who undergo complete resection, chemotherapy, and radiation have a 5-year survival of only 20%[2], underscoring the need for novel therapies. In

the year 2012, 43000 cases of pancreatic cancer were diagnosed and, as the general population continues to age, this incidence is expected to increase[3].

This review article discusses recent advances made in the treatment of pancreatic cancer, such as new chemotherapeutic regimens that have improved survival, the recognition of potentially pre-malignant lesions, the emergence of improved imaging modalities allowing early detection of pancreatic masses, the growing practice of minimally invasive and robotic pancreatic surgery, and an improved understanding of the molecular changes contributing to pancreatic cancer development.

ADVANCES IN CHEMOTHERAPY REGIMENS

Few effective chemotherapeutic options exist for metastatic pancreatic cancer. Since the 1990s, gemcitabine has been considered the standard agent of choice, and, although multiple different agents have been evaluated in combination with gemcitabine or alone, few have demonstrated positive impact on survival in patients with advanced disease[4-9]. More recently, higher response rates have been observed with the FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, oxaliplatin) regimen and with the gemcitabine + nab-Paclitaxel regimen than with gemcitabine alone. In the Actions Concertées dans les Cancer Colo-Rectaux et Digestifs (ACCORD) 11 trial, 342 patients with metastatic pancreatic cancer were randomly assigned to receive either FOLFIRINOX or single-agent gemcitabine as first-line treatment for pancreatic cancer[10]. Endpoints included overall survival, progression-free survival, tumor response (RECIST criteria), safety, and quality of life. On interim analysis, a significantly improved median overall survival was observed in the FOLFIRINOX arm (11.1 mo *vs* 6.8 mo, HR = 0.57, $P < 0.001$) compared with the gemcitabine arm[10]. However, there were significantly more grade 3-4 toxicities, such as cytopenias, diarrhea, and neutropenic fever, in the treatment group (all $P < 0.01$). Subsequent studies have confirmed the efficacy of the FOLFIRINOX regimen[11,12], but have questioned its applicability to patients of older age, with poor performance status, and with co-morbid conditions[13]. FOLFIRINOX also remains controversial with respect to its tolerability; studies report manageable side effects as well as significant toxicity resulting in treatment discontinuation[11,14]. In small studies, components of the FOLFIRINOX regimen have been dose-attenuated, raising the concern that physician modification of the regimen may affect patient outcomes[15].

Favorable outcomes are also beginning to be observed with the use of this regimen in the neoadjuvant setting for patients with borderline resectable or locally unresectable disease. In a recent study of 21 patients with either unresectable or borderline resectable pancreatic cancer who received neoadjuvant FOLFIRINOX, a 33% R0 resection rate was achieved (55% borderline resectable, 10% locally unresectable) and 24% of patients demonstrated a significant pathologic response[16]. Despite concerns of toxicity and tolerability, in the carefully selected patient with good performance status and early or advanced disease, FOLFIRINOX demonstrates potential for improved oncologic outcomes.

Nab-paclitaxel (trade name, Abraxane) is a nanoparticle albumin-bound (nab) paclitaxel that was initially developed to avoid hypersensitivity reactions resulting from solvents used to dissolve the agent[17]. It was approved by the FDA in 2004 for use in metastatic breast cancer and metastatic non-small cell lung cancer[18,19]. In the phase Ⅲ Metastatic Pancreatic Adenocarcinoma Clinical Trial (MPACT), the addition of nab-paclitaxel to gemcitabine demonstrated improved median overall survival (8.5 mo *vs* 6.7 mo, HR = 0.72, $P < 0.001$), improved 1-year survival (35% *vs* 22%), improved 2-year survival (9% *vs* 4%), and improved objective response rate (23% *vs* 7%) when compared with gemcitabine alone[20]. Although this response is not as dramatic as those observed with FOLFIRINOX, this regimen was well-tolerated and demonstrated a safer toxicity profile. It has emerged as an option for patients who cannot tolerate FOLFIRNOX because of poor performance status.

The role of nab-paclitaxel was investigated in pancreatic cancer after molecular profiling done on pancreatic tumors demonstrated high levels of the albumin-binding protein SPARC (secreted protein acidic and rich in cysteine)[21]. Nab-paclitaxel has demonstrated anti-tumor activity in cancers of the breast and lung, particularly in tissues that express high levels of SPARC[22]. It is believed that among patients with pancreatic cancer, tumors with high SPARC expression serve as albumin-binding sites that sequester nab-paclitaxel and concentrate drug levels intratumorally[23]. Another mechanism proposed involves an albumin receptor (gp60) on endothelial cells that transports paclitaxel into the tumoral interstitial space[24].

Gemcitabine plus erlotinib is another multi-drug regimen that has shown improved progression-free survival and overall survival[25,26]. However, due to their greater potential for improved outcomes, FOLFIRINOX and gemcitabine + nab-paclitaxel are the preferred treatment options for patients with acceptable performance status.

EARLY IDENTIFICATION AND TREATMENT OF PREMALIGNANT LESIONS

Early detection and management of adenomatous polyps, in situ lesions, and other premalignant or potentially malignant entities of the colon and breast have resulted in less mortality due to these cancers. It is now believed that pancreatic ductal adenocarcinoma also arises from a series of similar progressive genetic mutations and specific precursor lesions, such as pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms.

PanINs are by far the most common of these precur-

sor lesions[27]. Autopsy studies have shown that panINs increase with age, are more common in the head of the pancreas, and are seen at much higher rates in pancreata with tumors than those with pancreatitis[28-30]. They involve the same molecular events seen in the development of adenocarcinoma of other organs, such as activation of K-ras mutants, overexpression of p53, and loss of p16 and SMAD4[31]. Although no specific sequence has been elucidated, certain mutations (K-ras, p16) occur before others (TP53, SMAD4), and higher grades of panIN indicate higher levels of mutations[32].

IPMN belong to a heterogenous group of cystic lesions and are also considered precursor lesions for the development of invasive carcinoma. Main-duct IPMNs connect to the main duct of Wirsung while side-branch IPMNs originate from smaller branches off the main duct. Main duct and branch duct IPMNs were associated with malignancy in 70% and 25% of cases, respectively. Other groups have produced similar findings[33]. There is a strong consensus for resection of main-duct IPMNs due to their higher risk for associated malignancy.

Mucinous cystic neoplasms comprise around 25% of all resected cystic neoplasms[34]. They are characterized by dense stroma surrounding a tumor with mucin-producing epithelial cells, which are susceptible to various degrees of atypia. In a study of mucinous cystic neoplasms by the Massachusetts General Hospital, the risk of malignancy among 163 cases was 17.5%, and all malignant tumors had either nodules or were greater than 4 cm in size[35]. Patients are typically managed by surgical resection. If non-operative management is pursued, lifelong surveillance is essential.

With the widespread use of radiographic imaging and improvement in its resolution, there has been an increase in the incidence of cystic lesions, which are now found in approximately 1% of all abdominal computed tomographic scans obtained[36]. Given the variable potential for malignancy, groups have developed criteria to characterize these lesions and risk-stratify patients. The diagnostic algorithm often includes endoscopic ultrasound (EUS) with fine needle aspiration of cyst fluid to assess cytology, the presence of mucin, tumor markers carcinoembryonic antigen, and DNA for loss of heterozygosity and K-ras mutations.

High resolution endoscopic ultrasound (EUS) is an imaging modality that is able to detect focal lesions as small as 2-3 mm in size[37]. Studies have shown that EUS is superior or at least equal to computed tomography (CT) or magnetic resonance imaging in its sensitivity for detecting lesions, determining tumor size and extent, and assessing lymph node involvement and vascular invasion[38,39]. Conventional CT scans also provide detailed high-resolution views of pancreatic tumors in relation to the superior mesenteric artery, celiac axis, superior mesenteric vein, and portal vein, and this imaging modality remains the preferred choice for initial evaluation of most patients suspected to have pancreatic cancer.

Early detection of pre-malignant and potentially malignant lesions represents a significant advance in the treatment of pancreatic cancer. Since invasive pancreatic cancer is rarely cured, resection of these premalignant lesions is believed to be warranted. However, further refinements in our understanding of premalignant lesions and more accurate risk-stratification of patients is necessary so that patients with a low risk of malignancy can avoid an operation.

ADVANCES IN SURGICAL PRACTICE

Surgery plays a critical role in the management of pancreatic cancer, and many advances in surgical practice patterns as well as surgical technique have resulted in reduced perioperative morbidity and mortality. Centralization of pancreaticoduodenectomy, for example, to higher-volume centers with higher-volume surgeons, has contributed to a reduction in postoperative mortality, such that the risk of mortality at high volume centers is currently as low as 3%[40].

Historically, pancreatic tumors were considered either resectable or unresectable. In 2003, the National Comprehensive Cancer Network introduced the "borderline resectable" classification for pancreatic cancer, which refers to tumors that are involved with nearby structures so as to be neither clearly resectable nor clearly unresectable[41]. Aggressive management of this group of patients with neoadjuvant chemotherapy has made surgery feasible and may have improved survival in selected patients. The safety of vascular reconstruction in conjunction with pancreaticoduodenectomy has also been demonstrated in a systematic review of the literature[42].

Aside from more complex open surgeries, pancreatic cancer is also being increasingly approached laparoscopically. Early studies show that minimally invasive approaches can be performed safely and facilitate shorter hospital stay, earlier return to preoperative activity level, and reduced postoperative recuperation allowing for less delay in time to adjuvant chemotherapy or radiation[43-45]. With evolving technology and experience, laparoscopic distal pancreatectomy has become a standard approach for benign and malignant lesions of the pancreatic body and tail. In a multicenter study comparing open and laparoscopic distal pancreatectomy for patients with pancreatic ductal adenocarcinoma, Kooby *et al*[46] showed that there were no significant differences in positive margin rates, number of nodes examined, number of patients with at least one positive node, or overall survival, and that there was shorter hospital stay (7.4 d *vs* 9.4 d, $P = 0.06$) in the laparoscopic distal pancreatectomy group.

Laparoscopy has been extended to pancreaticoduodenectomy as well, and several case series have demonstrated feasibility, safety, and efficacy of this approach as compared to open surgery[47]. The robotic platform is also being increasingly adopted in pancreatic surgery. This approach overcomes limitations of laparoscopy, such as two-dimensional visualization, lack of dexterity, and poor ergonomics. In a series of 30 patients undergoing robot-

assisted major pancreatectomy and reconstruction, Zureikat *et al*[48] reported an overall pancreatic fistula rate of 27% and a 90-d Clavien grade Ⅲ-Ⅳ complication rate of 23%. They concluded that robot-assisted surgery can be performed safely with postoperative complication rates comparable to those of open pancreatectomy[48]. Further experience and larger, controlled studies are needed to clearly define potential benefits and elucidate long-term oncologic outcomes of minimally invasive pancreaticoduodenectomy.

ADVANCED UNDERSTANDING OF GENETIC AND MOLECULAR FACTORS

Many genetic alterations, including germ line and somatic mutations, contribute to the development of pancreatic cancer. Recent studies indicate that pancreatic cancer cells carry an average of 63 genetic mutations per cancer, and these mutations can be grouped into twelve core signaling pathways[49]. Over 90% of pancreatic cancers possess mutations in the k-ras oncogene, which is mutated in 20%-30% of all human malignancies[50]. Mutations within this oncogene are most often located on exon 1 of codon 12 and sometimes on codons 61 and 13[50,51]. Mutated k-ras upregulates several pathways, such as the PI3K-AKT pathway, which is involved in a series of important cellular functions, including survival and proliferation[52]. Other oncogenes involved in pancreatic carcinogenesis include those involved with the Notch signaling pathway[53] and the sonic hedgehog pathway[54].

The most widely recognized tumor suppressor gene (TSG) implicated in pancreatic cancer development is p53, which is found to be mutated in over 75% of specimens[55]. Other TSGs of importance include DPC4 (Deleted in Pancreatic Cancer, locus 4), LKB1 (liver kinase B1), p16, MAPK (mitogen activated protein kinase), and BRCA 2. These various discoveries contribute to the development of more targeted therapies and may also provide prognostic information. Over 50% of pancreatic adenocarcinomas have been demonstrated to have an inactivating mutation in SMAD4[56]. Tascilar *et al*[56] measured SMAD4 protein expression in 249 pancreatic adenocarcinomas and found that patients with this mutation had significantly longer survival than those without it (19.2 mo *vs* 14.7 mo), even after adjusting for other factors such as tumor size, margins, lymph node status, pathological stage, blood loss, and use of adjuvant chemoradiotherapy.

Whole exome sequencing and copy number analysis of a prospective cohort of 142 patients with pancreatic cancer recently defined 16 significantly mutated genes, ranging from those which were previously known to contribute to pancreatic cancer pathogenesis (KRAS, TP53, CDKN2A, SMAD4, MLL3, TGFBR2, ARID1A, SF3B1) to newly discovered genes involved in chromatin modification (EPC1, ARID2), DNA damage repair (ATM), and other mechanisms (ZIM2, MAP2K4, NALCN, SLC16A4, MAGEA6)[57]. Larger studies are needed to determine whether these mutations are more prevalent among specific demographic groups or whether they affect oncologic outcomes.

CONCLUSION

Pancreatic cancer remains a highly lethal disease. By the time patients are diagnosed, the disease may often be advanced, precluding patients from surgery. Recent advances in chemotherapeutic regimens have not only improved our ability to treat patients with metastatic disease, but have also shown favorable outcomes in the neoadjuvant setting. Advances in imaging technology and a better understanding of the pathogenesis of pancreatic cancer are allowing earlier diagnosis and early aggressive management of potentially pre-malignant entities. Emergence of high volume centers, the incorporation of imaging technology, and the availability of specialty services, such as interventional radiology, have reduced perioperative morbidity and mortality associated with pancreaticoduodenectomy. Furthermore, advances in surgical technology are allowing these procedures to be performed in less invasive fashion and are demonstrating safety and feasibility. Despite these advances, there remains room for improvement. Today's pancreatic oncologists must focus on further understanding the genetic and molecular factors contributing to oncogenesis and on the development of more targeted and less toxic systemic therapies.

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2014 ADVANCES IN PANCREATIC CANCER

Cachexia and pancreatic cancer: Are there treatment options?

Tara C Mueller, Marc A Burmeister, Jeannine Bachmann, Marc E Martignoni

Tara C Mueller, Jeannine Bachmann, Marc E Martignoni, Department of Surgery, University Hospital Rechts der Isar, Technical University of Munich, 81675 Munich, Germany

Marc A Burmeister, Braun Melsungen AG/Department of Anesthesiology, University Hospital Hamburg Eppendorf, 20246 Hamburg, Germany

Author contributions: Mueller TC and Burmeister MA contributed equally to this work; They performed the literature search and wrote the manuscript; Trial results and their clinical relevance were discussed with Martignoni ME and Bachmann J; Furthermore, Martignoni ME and Bachmann J revised and edited the manuscript; all authors were involved in conception and design of the manuscript.

Correspondence to: Marc E Martignoni, Professor, Department of Surgery, University Hospital Rechts der Isar, Technichal University of Munich, Ismaninger Strasse 22, 81675 Munich, Germany. martignoni@tum.de

Telephone: +49-89-41405093 Fax: +49-89-41404870
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Abstract

Cachexia is frequently described in patients with pancreatic ductal adenocarcinoma (PDAC) and is associated with reduced survival and quality of life. Unfortunately, the therapeutic options of this multi-factorial and complex syndrome are limited. This is due to the fact that, despite extensive preclinical and clinical research, the underlying pathological mechanisms leading to PDAC-associated cachexia are still not fully understood. Furthermore, there is still a lack of consensus on the definition of cachexia, which complicates the standardization of diagnosis and treatment as well as the analysis of the current literature. In order to provide an efficient therapy for cachexia, an early and reliable diagnosis and consistent monitoring is required, which can be challenging especially in obese patients. Although many substances have been tested in clinical and preclinical settings, so far none of them have been proven to have a long-term effect in ameliorating cancer-associated cachexia. However, recent studies have demonstrated that multidimensional therapeutic modalities are able to alleviate pancreatic cancer-associated cachexia and ultimately improve patients' outcome. In this current review, we propose a stepwise and pragmatic approach to facilitate and standardize the treatment of cachexia in pancreatic cancer patients. This strategy consists of nutritional, dietary, pharmacological, physical and psychological methods.

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Key words: Cachexia; Pancreatic neoplasms; Nutritional support; Gastrointestinal neoplasms

Core tip: Cachexia in pancreatic cancer is frequently described and reduces survival and quality of life of the concerned patients. Despite intense pre-clinical and clinical research activities, there are still no pharmaceutical agents with proven effectiveness in the long term. Furthermore, it is evident that only multimodal concepts can improve patients' outcome. Therefore, the current pharmacological and nutritional therapy options are reviewed and a stepwise and pragmatic approach will be presented to facilitate and standardize the treatment of cachexia in pancreatic cancer patients. This strategy combines nutritional, dietary, pharmacological and as well physical and psychological methods.

INTRODUCTION

Cachexia, definition and diagnostic criteria

Cachexia is a multi-factorial, systemic syndrome characterized by pathological weight loss due to excessive wasting of skeletal muscle and adipose tissue mass. It can occur in the course of chronic benign diseases like chronic heart failure or chronic obstructive pulmonary disease (COPD) as well as in the course of infectious diseases like tuberculosis or human immunodeficiency virus (HIV)-infection. However, it is most frequently observed concomitantly with malignancies, especially in pancreatic- and lung cancer. The mechanisms that lead to cachexia are still poorly understood, but consensus is that is has to be regarded as a complex of multiple, interactive patient- and tumor-specific components, such as metabolic and humoral changes as well as psychological issues, anorexia, fatigue and adverse effects of anticancer therapies (Figure 1).

In cancer patients, the presence of cachexia is associated with poor prognosis, reduced treatment tolerance and a marked reduction in quality of life (QoL)[1,2]. Therefore, the preservation of lean body mass (LBM) is critical for cancer patients, but despite all efforts an effective treatment for cachexia is still lacking.

The diagnostic criteria for cancer cachexia are still not very strictly defined. In addition, weight loss is multi-factorial and can be difficult to assess. In particular the loss of skeletal muscle tissue can be hard to quantify, especially in obese individuals. These problems combined with the use of different diagnostic criteria by different research groups in the past, has led to heterogeneity in clinical and experimental trials. Therefore, an international consensus regarding the definition of cachexia was made in 2011. According to this consensus, cachexia is defined by unintended weight loss of more than 5% of body weight or weight loss of more than 2% in individuals with a body mass index (BMI) of less than 20 kg/m^2, over 6 mo. Additionally, the presence of sarcopenia (skeletal muscle depletion) with any degree of weight loss of more than 2% should be classified as cachexia[3]. Sarcopenia can be detected by the following methods of assessment: anthropometry of mid-upper-arm muscle area (men < 32 cm^2, women < 18 cm^2), appendicular skeletal muscle index determined by dual-energy X-ray absorptiometry (men < 7.26 kg/m^2, women < 5.45 kg/m^2), lumbar skeletal-muscle index determined from oncology computed tomography (CT) imaging (men < 55 cm^2/m^2, women < 39 cm^2/m^2), and whole-body fat-free mass index without bone determined by bioelectrical impedance (men < 14.6 kg/m^2, women < 11.4 kg/m^2)[4].

Furthermore, reduced food intake, anorexia, markers of systemic inflammation like C-reactive protein (CRP), responsiveness to chemotherapy and disease progression should be assessed for the diagnosis of cancer cachexia[2,3]. However, studies published after 2011 still frequently use their own diagnostic criteria or cut off values for cachexia. New techniques, like measuring different body tissue masses based on a CT-scan may ultimately facilitate the standardization of diagnosis of cachexia in the future[5]. Furthermore, this method allows for the quantification of occult tissue loss in muscle, subcutaneous- and visceral adipose tissue (VAT), even in obese patients. It has been shown that not only the degree of weight loss impacts survival of pancreatic cancer patients, but also the proportion of muscle and fat loss in the different compartments[6,7]. To perform the assessment, cross-sectional areas of the left and right psoas muscles at the level of the fourth lumbar vertebra (L4) can be used. The surface is usually expressed in square millimeter[8]. Studies using these CT-image based techniques show that the loss of muscle tissue is particularly associated with a shorter survival in cancer patients[5]. Congruently, a recent study in which body tissue mass was measured by CT scans in pancreatic cancer patients, was able to show that sarcopenia in obese patients is an occult condition, associated with a shorter survival[7].

Cachexia in pancreatic cancer: incidence, impact on prognosis and outcome

In Western countries, pancreatic ductal adenocarcinoma (PDAC) is among the top five causes of cancer deaths[9]. Unfavorable prognosis of this cancer entity can be attributed to late diagnosis and aggressive tumor biology and affection other organ systems due to the function and anatomical location of the pancreas. Furthermore, among all malignancies patients with PDAC have the highest incidence of cancer cachexia and experience severe symptoms of this syndrome[6,10-13]. Cachexia has been shown to be present in up to 70%-80% of patients with PDAC and is associated with reduced survival, more progressive disease and higher rates of metastatic disease[12-15]. The presence of cachexia was shown to worsen the post-operative outcome of patients with pancreatic cancer[15,16]. However, currently the only hope for cure of pancreatic cancer is the complete surgical resection of the tumor, which is only possible in non-metastatic and locally restricted stages.

Especially the loss and the rate of loss of VAT tissue seems to be correlated with a worse prognosis in pancreatic cancer patients, possibly due to the metabolic activity of this tissue[17]. Moreover, an association of VAT-loss with the presence of diabetes and anemia was observed in these patients[6]. Alterations in metabolism and a systemic inflammatory reaction contribute largely to the wasting of muscle and adipose tissue in pancreatic cancer. A central role in the development and regulation of cachexia in pancreatic cancer is attributed to the inflammatory response in the liver[18]. The acute phase response in the liver is characterized by the production of inflammatory compounds like CRP as well as the *de novo* synthesis of pro-inflammatory cytokines like interleukin (IL)-6, IL-1β, IL-8 and tumor necrosis factor (TNF)-α. Additionally, high quantities of these cytokines are produced by peripheral mononuclear cells and pancreatic cancer cells[18,19]. These pro-inflammatory mediators not
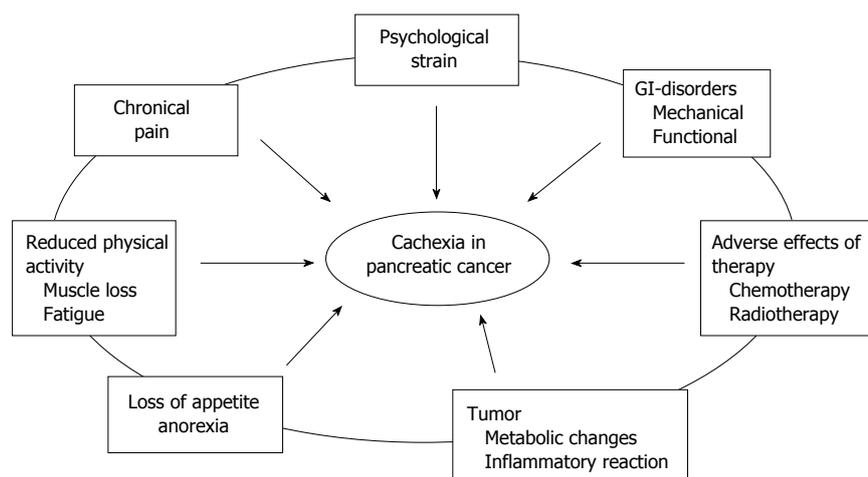
Figure 1 Multifactorial genesis of cachexia in pancreatic cancer. GI: Gastrointestinal.

only maintain the acute phase response in the liver, but also have effects on the central nervous system that lead to anorexia and fatigue. It was shown that patients with progressive weight loss exhibit increased levels of pro-inflammatory cytokines, which enhance lipid and protein catabolism whereas anabolic pathways [*e.g.*, IGF-1/Akt/ mammalian target of rapamycin (mTOR)] seem to be inhibited[6,19-21]. In pancreatic cancer patients, elevated levels of these cytokines were associated with poor performance status and weight loss[22]. Accordingly, IL-1 and IL-6 gene polymorphisms have been shown to be associated with a higher incidence of cachexia and shortened survival in pancreatic cancer patients[23].

Moreover, neuro-endocrine hormones (*e.g.*, leptin, neuropeptide Y, corticotropin-releasing factor, melano-cortin, neurotensin) and tumor-derived factors such as proteolysis-inducing factor or lipid mobilizing factor contribute to tissue catabolism and appetite regulation in a complex interaction which is not yet fully understood[12]. Finally, secondary symptoms of pancreatic cancer like chronic pain, nausea and pancreatic insufficiency additionally reduce appetite and food intake[24].

CURRENT TREATMENT OPTIONS OF CACHEXIA IN PANCREATIC CANCER PATIENTS

As mentioned above, the best way to treat pancreatic cancer patients is to surgically resect the tumor. However, less than 15% of patients are eligible for surgery at first presentation[25], and only approximately 70% of these tumors are fully resectable at the operation[14]. Palliative treatment of non-resectable pancreatic cancer consists of chemotherapy, radiotherapy (not routinely) and supportive care. An essential element of supportive care is the preservation of QoL. It was shown that cachexia substantially reduces QoL in pancreatic cancer patients. In addition, cachexia is exacerbated by systemic chemotherapy and decreases its tolerance[4,10]. Hence, treating

cachexia and stabilizing weight can be crucial for patients with pancreatic cancer and may prolong their survival[23].

In consistency with the multi-factorial pathogenesis of cachexia in pancreatic cancer, it is widely recognized that a multimodal treatment approach is necessary[1]. This includes nutritional support and exercise as measures to stabilize weight, as well as pharmacological treatment of inflammatory and metabolic changes, and the treatment of secondary symptoms that exacerbate cachexia such as loss of appetite, mechanical or functional impairment of the gastrointestinal tract, chronic pain, fatigue and depression[4].

However, there is currently no guideline on clinical management of cachexia in pancreatic cancer and - albeit extensive research - there is still no successful pharmacological treatment. In the following paragraphs, current treatment options will be discussed and a multimodal, stepwise approach will be presented (Figure 2).

Nutritional support for cachectic pancreatic cancer patients

Supportive nutrition and caloric supplementation are important components of supportive care for cachectic patients with pancreatic cancer[26]. Preferably nutrition should be delivered enteral to avoid the side effects of parenteral nutrition[27]. Cachectic patients should be supplemented with 1000-1500 calories per day (20-25 kcal/kg per day for bedridden and 25-30 kcal/kg per day for ambulatory patients) in form of a balanced essential amino-acid mixture, given between meals[27,28]. For an adequate enteral function, it is important that vitamin D and exocrine pancreatic insufficiency are treated by supplementation[26,28]. For a sufficient supplementation 2000 IE of pancreatic enzymes are needed per 1g of fat. Furthermore, other concomitant symptoms that affect appetite and food intake, like mechanical or functional gastrointestinal disorders as well as depression and fatigue need to be addressed.

Recent studies demonstrate a clear benefit of nutritional supplementation for patients with pancreatic can-
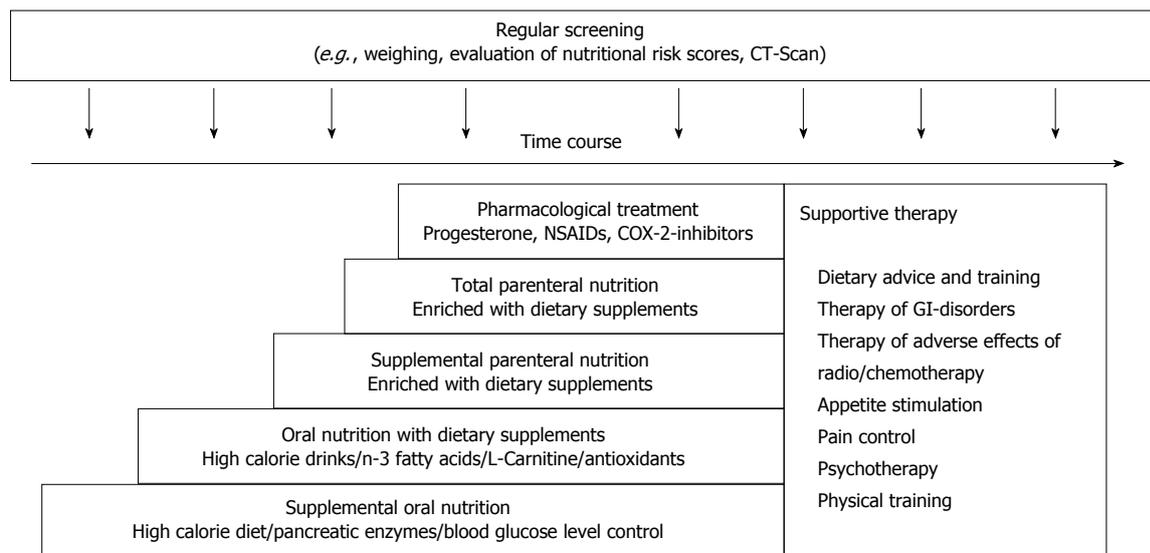
Figure 2 A stepwise, clinical approach for the treatment of cachexia in pancreatic cancer patients. GI: Gastrointestinal; NSAIDs: Non-steroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; CT: Computed tomography.

cer. For example, a study using CT-imaging to monitor body tissue loss showed that independent of the disease stage, pancreatic cancer patients who received any type of nutritional supplementation lost less muscle tissue compared to those receiving no nutritional supplementation. Moreover, survival time was increased in patients which received nutritional supplementation[6]. However, the number of patients included in this study was low and a variety of different nutritional products were used. Another study of palliative pancreatic cancer patients demonstrated that compliance with oral nutrition prescription improved energy/protein intake and weight stabilization[29]. Furthermore, it was shown that nutrition intervention together with chemotherapy improved outcomes and QoL of patients with pancreatic or lung cancer, without inhibiting meal intake[30]. Additional parenteral nutrition for cachectic pancreatic cancer patients also showed improvements in BMI, phase angle and ratio of extracellular mass to body cell mass[31].

However, to which extend or in which combination oral or parenteral nutritional support should be provided is still under extensive discussion. According to the ES-PEN-guidelines (European Society for Clinical Nutrition and Metabolism) parenteral nutritional support is indicated if inadequate food and enteral intake (< 60% of estimated energy expenditure) is anticipated for more than 10 d. Other indications are severe mucositis, radiation enteritis or intestinal failure and peri-operative support of cachectic patients. Parenteral nutrition should not be used and is probably harmful in well-nourished patients with adequate oral food intake[27].

DIETARY SUPPLEMENTS AND CANCER CACHEXIA

In addition to the simple supplementation of calories, specific nutrients can be administered to fight cachexia in pancreatic cancer patients. The most frequently tested dietary supplements in the treatment of cancer cachexia are summarized in Table 1.

N3-fatty acids like eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), which are largely contained in fish oil, have been studied intensively as additional treatment for cancer cachexia[32-35]. N3- or omega-3 polyunsaturated fatty acids have been shown to modulate levels of pro-inflammatory cytokines, hepatic acute phase proteins, eicosanoids, and tumor-derived factors in animal models of cancer cachexia[12]. EPA and DHA are metabolized by cyclooxygenase (COX) and 5-lipoxygenase yielding in metabolites with less inflammatory and immunosuppressant potency than the substances derived from arachidonic acid[12]. Moreover, it was shown that EPA induced apoptosis in three different pancreatic cancer cell lines and inhibited cell growth in a dose-dependent manner[36]. N3-fatty acids are meanwhile ingredients of most enteral and in some parenteral supplements. However, in oral nutritional support the doses needed to achieve an effect are high and a large amount of product needs to be consumed, which can be problematic for cachectic patients.

Another dietary supplement that has been proposed for the treatment of cachexia treatment is L-Carnitine. L-Carnitine is required to transport long-chain fatty acids, as a major source of energy, into the mitochondrial matrix for β-oxidation. Highest levels of L-Carnitine are observed in skeletal and cardiac muscle. It has been suggested that a deficiency of L-Carnitine contributes to cachexia in cancer patients[37,38]. In animal models, supplementation of L-Carnitine resulted in a significant improvement of food intake, muscle weight and physical performance. On the molecular level L-Carnintine administration decreased proteasome activity and related gene-expression, as well as the expression of genes involved in apoptosis. In addition, it was shown that *in vitro*

Table 1 Dietary supplements in the treatment of cancer cachexia

Agent	Mechanism of action	Ref.
N3-fatty acids (EPA, DHA, fish oil)	Reduction of pro-inflammatory cytokines and acute-phase-response	[32-36]
L-Carnitine	Antioxidant, cofactor of mitochondrial production of Acetyl-coA (ß-oxidation, aminoacid metabolism)	[37-40]
Antioxidants	Reduction of ROS-formation and oxidative stress	[42-45]
(GSH, ALA, NAC, vitamins A/C/E)		
Branched-chain-amino acids	Anabolic effects, stimulation of appetite and food intake	[46-49]
Lactoferrin	Increase of hemoglobin in anemic patients, iron-metabolism, decrease of inflammatory response	[50]

EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; GSH: Glutathione; ALA: Alpha-lipoic acid; NAC: N-acetyl-cysteine.

application of L-Carnitine to muscle cells resulted in a direct decrease of the proteolytic rate[39]. Clinical trials have been reviewed by Silvério *et al*[38] in 2011. A recent randomized multicenter trial included 72 patients with pancreatic cancer and compared patients who received 4 g oral L-Carnitine for 12 wk to a placebo group. An increase in body weight, QoL and a trend towards increased overall survival was observed in the L-Carnitine-treated group[40].

Oxidative stress and the formation of reactive oxygen species (ROS) play an important role in the pathogenesis of cancer cachexia and represent another potential target for intervention. Mechanisms that lead to the accumulation of ROS are mainly the lack of natural antioxidants due to reduced food intake and the chronic inflammatory reaction. The formation of ROS is further exacerbated by the use of alkylating chemotherapeutic agents such as cisplatin[41,42]. Exogenous antioxidants are vitamins A, C, E and polyphenols. Endogenous antioxidants are a range of enzymes, especially glutathione peroxidase, as well as glutathione, alpha-lipoic acid (ALA), N-acetyl cysteine, reduced coenzyme Q10, melatonin, and plasma protein thiols[41,43]. A few clinical trials showed that antioxidants reduced levels of ROS and pro-inflammatory cytokines in advanced cancer patients[44]. However, in a recent study on melatonin as treatment for cancer cachexia it was shown that there was no improvement of weight, QoL or appetite in patients with advanced cancer[45].

Other dietary supplements in cachexia treatment include branched chain amino acids like valine, leucine and its metabolite β-hydroxy-β-methylbutyrate, which have anabolic effects on skeletal muscle mass. Experimental data suggests that they enhance protein anabolism and improve appetite and food intake in cancer cachexia[46,47]. However, results from clinical trials have been rather disappointing so far and have not yet led to a recommendation towards their use alone or in combination protocols[48,49].

Another nutritional supplement with potentially beneficial effects on cachexia is lactoferrin. In a recent clinical trial it was demonstrated that supplementation of lactoferrin was able to ameliorate cancer-associated anemia in patients with advanced stage (Ⅲ/Ⅳ) solid, malignant tumors (gynecological, colon, stomach, prostate, bladder, lung). Furthermore, there was a decrease of serum levels of inflammatory markers in the lactoferrin-treated arm[50].

Even though a large amount of clinical studies have investigated the effects of these dietary supplements in the treatment of cancer cachexia, many of which observed positive effects, the overall results remain inconclusive for a definite recommendation on their use in clinical practice. This is also due to the fact that design, products and used definitions of cachexia vary largely between the trials, a problem encountered generally in clinical trials of dietary supplements[51]. However, some of the trials specifically in patients with pancreatic cancer show promising results and should be verified in larger and standardized clinical trials.

PHARMACOLOGICAL TREATMENT OF CACHEXIA IN PANCREATIC CANCER PATIENTS

Pharmacological treatment of cachexia includes drugs that improve appetite, the treatment of secondary symptoms that enhance cachexia, and newer drugs that specifically target the molecular mechanisms involved in the pathogenesis of cachexia[26,52]. The current pharmacological approaches are summarized in Table 2. Although more and more drug targets are proposed based on extensive research in animal models, so far very few pharmacological treatments have been translated into clinical practice and there is no single pharmacological treatment that successfully and consistently ameliorates cachexia in pancreatic cancer patients.

Appetite stimulation
Drugs that ameliorate appetite and food intake are an important component of cachexia therapy in cancer patients, since the majority of them suffer from anorexia. Drugs containing the active ingredient of cannabis (Tetrahydrocannabinol, THC) like dronabinol have been used to fight chemotherapy related nausea and anorexia in the past. The endocannabinoid system plays an important role in energy homeostasis. However, results of trials investigating the role of cannabis extracts in the treatment of cancer induced cachexia have been disappointing in terms of weight gain, although improvements in appetite and mood were observed in some studies[53]. Furthermore, there are significant side effects of this treatment, which is why it is currently not recommended in Europe. These include impairment of cognitive function, mental confusion and somnolence and may enhance depression and other psychiatric disorders[41].

Table 2 Pharmacological treatment approaches for cancer cachexia

	Agent	Mechanism of action	Ref.
Potentially effective therapies	Progesterone (MA, MPA)	Appetite stimulation through neuropeptide y down-regulation of pro-inflammatory cytokines	[59,61,90-92]
	Corticosteroids	Inhibition of prostaglandin activity, suppression of IL-1 and TNF-α	[62]
	Anabolic androgens	Muscle anabolism, up-regulation of protein synthesis, dose-dependent alterations of Akt-phosphorylation, GLUT-4 and ISR-expression	[64,65]
Experimental therapies	SARMs	Selective modulation of androgen receptors in muscle tissue only	[67-69]
	NSAIDs	Inhibition of COX-1 and -2 prostaglandin-synthesis, decrease of inflammatory reaction	[71,72]
	COX-2 selective inhibitors	Inhibition of prostaglandin-synthesis, decrease of inflammatory reaction, additional antineoplastic and anti-angiogenetic effects	[70,73,92]
	Thalidomide	Inhibition of TNF-α, and other pro-inflammatory cytokines, NF-κB, inhibition of COX-2	[74-76]
	Anti-TNF mAb	Inhibition of TNF-α	[78,79]
	Anti-IL-6 mAb	Inhibition of IL-6	[85]
	ACE-Inhibitors	Inhibition of angiotensin converting enzyme, role in cancer cachexia not yet fully understood	[88,89]
	Myostatin-inhibitors/ Act II rb-antagonists	Inhibition of Act II rb signaling, stimulation of muscle growth and regeneration	[4,66,86,87]
	Ghrelin/Ghrelin mimetics	Stimulation of GH-secretion, appetite stimulation though neuropeptide y, decrease of sympathetic nerve activity	[54-57]
	Mirtazepin, Olanzapine	Appetite stimulation through serotonergic blockade	[58,59]
Treatments without proven effectiveness	Pentoxifylline	Inhibition of TNF-α	[77]
	Insulin, IGF-1, GH	Regulation of body composition (fat, glucose and protein metabolism) *via* PI3K/Akt-, MAPK-pathways	[63,64,95]
	Cannaboids (dronabinol)	Appetite stimulation, energy hemostasis	[53]

MA: Megestrol acetate; MPA: Medroxyprogesterone acetate; COX-2: Cyclooxygenase-2; SARMs: Selective androgen receptor modulators; NSAIDs: Non-steroidal anti-inflammatory drugs; TNF: Tumor necrosis factor; IL: Interleukin; IGF: Insulin-like growth factor; GH: Growth hormone; GLUT-4: Glucose transporter-4; ISR: Induced systemic resistance; MAPK: Mitogen-activated protein kinase.

Another new approach to treat anorexia is to target the leptin/ghrelin/neuropeptide-γ axis. Ghrelin is a peptide hormone which is produced in the stomach and stimulates growth hormone (GH)-secretion and increases appetite through neuropeptide-γ system[52]. Ghrelin and the ghrelin receptor agonists (anamorelin and RC-1291) are currently in phase III clinical trials and show promising preliminary results in increasing food intake and body weight in cancer patients, with minimal adverse effects[54-57]. In addition, these positive effects were shown to be potentiated by the traditional Japanese medicine Rikkunshito, which stimulates endogenous ghrelin production[55]. However, results from clinical trials are not univocal in terms of efficacy, dose prescription and more research is needed.

Neuroleptic drugs like mirtazapine and olanzapine are often used to treat chemotherapy-induced nausea through serotonic blockage. In addition, they increase appetite which is why they have been proposed as additional treatment of anorexia in cancer cachexia[58]. Furthermore, they might have positive effects on pro-inflammatory cytokine levels. However, the mechanisms of action are not fully understood and clinical trials are needed to evaluate their effect on cancer cachexia, specifically. A trial comparing treatment with the progesterone megestrol acetate (MA) in combination with olanzapine was more effective than MA alone in cachectic patients with advanced gastrointestinal or lung cancer (stage III/IV)[59].

Progesterones, corticosteroids and anabolic hormones

Progesterones represent another pharmacological approach. The mechanism of progesterone action is to stimulate appetite through direct and indirect pathways in the central nervous system. In addition, it is suggested that they antagonize the catabolic effects and downregulate the production of pro-inflammatory cytokines[12]. Synthetic progesterones such as MA and medroxyprogesterone acetate (MPA) have been shown to significantly improve appetite and partially reverse fat loss in randomized controlled trials but failed to improve global QoL or survival in most cancer cachexia trials[41,60,61]. In a recent updated meta-analysis (35 trials, including almost 4000 patients) it has been shown that, compared to a placebo group, treatment with MA improved appetite, weight gain and QoL in patients suffering from cachexia due to cancer, HIV/AIDS or other pathologic conditions. However, significant side-effects were observed, in particular thromboembolic complications and edema[61]. Therefore, a careful and individual risk/benefit analysis should be performed before its application in cachectic patients with pancreatic cancer. Furthermore, the optimal dose for prescription of MA remains to be determined. In clinical practice, MA is often combined with corticosteroids and some older clinical trials also reported possible benefits of the combination of MA with ibuprofen in cancer patients[12].

Corticosteroids (*e.g.*, prednisolone, methylprednisolone) inhibit prostaglandin activity and suppress pro-inflammatory cytokines like IL-1 and TNF-α. Furthermore, there are central effects leading to improved appetite and euphoria. However, the effects generally don't last longer than 2-4 wk and long-term corticosteroid therapy is associated with substantial adverse effects like dysmetabolism, osteoporosis, myopathy and an increased risk of infec-

tions[12]. There are only a few, older trials that specifically evaluated corticosteroids in cancer cachexia. A recent randomized double blind study indicated that treatment with dexamethasone in patients with advanced cancer (all types of solid tumors) ameliorated fatigue and QoL[62].

It is widely recognized that during cachexia, signaling of insulin, insulin-like growth factor-1 (IGF-1) and GH is dysregulated. GH normally induces the production of IGF-1 in the liver and other tissues. IGF-1 stimulates protein synthesis, myoblast differentiation, and muscle growth, whereas it suppresses protein degradation. The dysregulation of this axis causes an anabolic/catabolic imbalance which leads to loss of LBM[63]. In cachetic patients low serum concentrations of IGF-1 have been observed, while there seems to be a peripheral GH and Insulin resistance, which leads to a negative protein balance, especially in skeletal muscle tissue. The United States Food and Drug Administration currently approved recombinant GH for treatment of muscle wasting in HIV/AIDS, parenteral nutrition-dependent short bowel syndrome, and pediatric chronic kidney disease[64]. However, the therapeutic application of Insulin, GH or IGF-1 for pancreatic cancer patients is currently not recommended due to adverse effects (paresthesia, arthralgia, sodium retention and peripheral edema) of the high doses that would be required due to the peripheral insulin and GH-resistance[63,64]. New experimental therapies try to target post-receptor pathways of IGF-1, GH and insulin, like the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway. However, the oncogenic potential of cell growth promoting treatments has to be kept in mind, since alterations in the PI3K/Akt pathway are common in cancer[63].

Testosterone and its synthetic derivates (*e.g.*, nandrolone, oxandrolone) are anabolic steroid hormones. They increase muscle mass through upregulation of protein-synthesis. Furthermore, there is also interaction with the Insulin/IGF-1/GH system in terms of dose-dependent alterations of Akt-phosphorylation, glucose transporter-4 (GLUT-4) and insulin receptor-expression. Low doses of testosterone increase insulin sensitivity, while high doses increase insulin resistance[63]. In cachexia due to HIV/AIDS or COPD, treatment with testosterone has been shown to improve body weight and functional parameters, however there are only very few trials on its use in cancer cachexia[64,65]. Adverse effects reported are elevated transaminase levels, jaundice, virilization and decreased high density lipoprotein concentrations. Furthermore, there are many interactions with other medications, *e.g.*, oral anticoagulation. In addition, it has to be kept in mind that anabolic steroids potentially lead to fluid retention, which might cause false positive results in clinical trials on weight gain[64]. A more promising new approach is the treatment with SARMs. These molecules react with androgen-receptors in muscle tissue only, minimizing the systemic side effects of androgen therapy. Apparently, several pharmaceutical companies are currently testing these agents to fight sarcopenia due to aging and

cancer cachexia[66]. For example, Ostarine has demonstrated promising results in Phase I and II clinical trials and may have the ability to perform as a potent anabolic agent with minimal side effects[67]. Similarly, Enobosarm is currently being tested in phase II clinical trials[68,69]. However, larger clinical trials are warranted to confirm these preliminary results.

Anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) or selective cyclooxygenase-2 (COX-2)-inhibitors not only reduce the inflammatory response but also have a positive effect on resting energy expenditure (REE) and were shown to prolong survival in malnourished patients with advanced cancer (mostly gastrointestinal cancers)[70,71]. NSAIDs that have been evaluated in cancer cachectic patients are ibuprofen and indomethacin. A recent systematic review of 13 clinical trials found that all but two trials showed an improvement in body weight, physical performance and QoL, while side effects were extremely rare. However, the number of patients included in these trials is small and they used variable outcomes. Therefore, evidence is too frail to recommend the use of NSAIDs to treat cancer cachexia in clinical routine yet[72].

Selective inhibitors of COX-2 (*e.g.*, celecoxib) also received a lot of attention in the search for cachexia treatments. They reduce the systemic inflammatory reaction and there is evidence of anti-neoplastic properties in animal models[41]. Phase II clinical trials in cachectic cancer patients with solid tumors at different sites have shown an increase in LBM and QoL[73]. However, more clinical trials are needed to confirm these results.

Anti-cytokine strategies

Since pro-inflammatory cytokines, such as TNF-α and IL-6, play a prominent role in the pathogenesis of cachexia in pancreatic cancer, systemic inflammation remains an important area for novel therapeutic targets[74]. Results from animal models regarding anti-cytokine strategies provide evidence that targeting cytokine signaling can ameliorate cachexia, even though it has been widely accepted that this complex syndrome is not caused by only one single, specific cytokine[41].

However, most clinical trials of inhibitors of synthesis or activity of TNF-α have so far not proven to be effective in preserving lean body mass in cancer patients[60,74]. The drug thalidomide, which downregulates the production of TNF-α and other pro-inflammatory cytokines and inhibits NF-κB, COX-2 and angiogenesis, was shown to be effective in the treatment of cancer cachexia in patients with gastro-intestinal and pancreatic cancer[4,75]. However, thalidomide has strong adverse effects, which warrant a careful risk-benefit analysis. A recent meta-analysis concluded that evidence is not sufficient to recommend its routine use to treat cancer cachexia[76].

Pentoxifyllin, which is another inhibitor of TNF-α, failed to improve weight loss in cachectic cancer patients with different types of solid tumors[74,77]. Anti-TNF-α an-

tibodies such as infliximab and etanercept did not show any significant improvements in cachectic patients and were not well tolerated, either[78,79]. Finally, results from preclinical studies suggested a potential new treatment for cachexia by inhibiting TNF-α converting enzyme. However, some of these substances were patented but never achieved to pass phase Ⅱ clinical trials. Penna *et al*[74] have reviewed other patented substances that inhibit cytokines directly or *via* receptor modulation or inhibition of NF-κB in detail recently. Notably, none of these substances has been evaluated for their efficiency in ameliorating cachexia in pancreatic cancer in larger clinical trials so far.

Elevated IL-6 levels were quite consistently associated with weight loss and a reduced the rate of survival in cancer patients[18,22,80-82]. Iwase *et al*[83] even showed that IL-6 was elevated in cachectic patients, whereas TNF-α was not. Several studies showed that IL-6 was significantly over-expressed in pancreatic tissue, and serum levels were significantly elevated in cachectic compared to non-cachectic patients with pancreatic cancer[18,22,84]. Preclinical and clinical (phase Ⅰ and Ⅱ) studies performed on the IL-6 antibody ALD518 in patients with non-small cell lung cancer (NSCLC) demonstrated that this treatment has the potential to improve anemia, reduce cancer-related cachexia and ameliorate fatigue, while having minimal adverse effects[41,85]. However, further research is clearly needed in this regard, since despite some promising results of small clinical trials, there is currently no approved anti-cytokine treatment for cancer cachexia[74].

Emerging pharmacological therapies

Recently, the myostatin/ActRⅡb pathway is receiving more and more attention in cachexia research. Animal models have shown that targeting this pathway can lead to dramatic increases in muscle mass[86,87]. ActRⅡb-receptor and myostatin inhibitors are currently being evaluated in clinical trials of muscle wasting and degenerative disorders. Among the first agents developed for clinical settings are the monoclonal anti-myostatin antibodies LY2495655 and BYM338, which are currently undergoing phase Ⅱ trials in patients with NSCLC and PDAC[4]. Apparently, several pharmaceutical companies have currently explored this pathway as therapeutic target in aging and sarcopenia, but their results have not yet been published[66].

Another interesting recent finding was that in patients with cachexia related to congestive heart failure, treatment with angiotensin-converting enzyme (ACE) inhibitors caused an increase in both subcutaneous fat and muscle mass[88]. There is also some preliminary evidence that ACE inhibitors have the potential to ameliorate cancer cachexia, at least in NSCLC patients[89]. However, the exact role of angiotensin Ⅱ in human cancer cachexia remains to be determined.

Searching the clinical trials databases of the NIH in the US (clinicaltrials.gov) or the EMA in Europe (clinicaltrialsregister.eu) for cachexia in pancreatic cancer, revealed only a very limited number of current trials. Only two ongoing trials are testing new pharmaceutical agents at the moment. One of these is a phase Ⅱ trial in advanced or metastatic pancreatic cancer. The other trial is a randomized, double-blind, placebo-controlled multi-center study for treatment of cachexia in patients with stage Ⅳ NSCLC or stage Ⅲ/Ⅳ pancreatic cancer. Beside these two industry-sponsored trials there is one more ongoing trial sponsored by the Greater Glasgow Health Board. This pre-MENAC study investigates the feasibility of a multimodal exercise/nutrition/anti-inflammatory treatment for cachexia in non-operable stage Ⅲ/Ⅳ NSCLC and pancreatic cancer.

COMBINATION PROTOCOLS

In regard to the multi-factorial pathogenesis of cachexia in pancreatic cancer, more and more clinical trials are testing combination protocols of the above-mentioned dietary supplements and pharmaceutical interventions.

For example, a trial by Mantovani *et al*[90] compared 4 different treatments with a combination arm, recieving all 4 treatments (progesterones, EPA, L-Carnitine; thalidomide) over 4 mo in patients with advanced stage solid tumors at any site. The most effective treatment in terms of LBM gain, REE, fatigue, appetite, IL-6 levels and Eastern Cooperative Oncology Group performance status score was the combination regimen that included all agents. The same research group implicated a new combination protocol in a non-randomized trial. The 16-week treatment consisted of a diet with high polyphenol content, oral nutritional support enriched with n-3 fatty acids (EPA and DHA), MPA, antioxidant treatment with ALA and carbocysteine lysine salt, vitamins E, A and C, and celecoxib. This treatment resulted in a positive response with increase of LBM and QoL in patients with advanced stage solid tumors at any site. Furthermore, there was a decrease of ROS and pro-inflammatory cytokines. No adverse effects were observed[42].

Another phase Ⅲ randomized trial included 104 advanced-stage gynecological cancer patients and assigned them to receive either a combination of MA with L-Carnitine, celecoxib, and antioxidants or MA alone over 4 mo. It was demonstrated that the combination arm was more effective with respect to LBM, REE, appetite, fatigue and global QoL. The inflammation and oxidative stress parameters IL-6, TNF-α, CRP, and ROS decreased significantly in the combination arm, while no significant change was observed in the MA arm[91]. Similarly, another trial compared two combination treatment arms, with or without MA and found no superiority of additional MA administration[92].

MULTIMODAL THERAPY AND A STEPWISE APPROACH FOR CLINICAL PRACTICE

Considering the multidimensional background of cancer cachexia, it is more and more accepted that multimodal therapeutic approaches, including exercise, nutrient sup-

plementation, appetite stimulation and pharmacological intervention, have to be implemented and individually adjusted for patients at different stages of cachexia[4,93]. Successful surgical removal of the tumor and/or oncological treatments should be the starting point for rehabilitation of patients with cancer-associated muscle wasting[94].

Figure 2 shows a stepwise approach of multimodal therapy options. On the first level oral nutrition should be optimized by a high calorie diet, regulation of blood glucose levels and supplementation of pancreatic enzymes. Improving patients' metabolism by insulin or metformin treatment was shown to increase whole body fat (without counteracting muscle loss) and survival in initial study results[95]. If there is no response to these measures, oral nutrition should be supplemented with high calorie drinks, enriched with dietary supplements such as EPA, L-Carnitine and antioxidants. In case of insufficiency of food intake, supplemental parenteral nutrition should be considered. Only the next step would require total parenteral nutrition. A large-scale meta-analysis showed that nutritional interventions were successful in increasing energy intake, body weight and some aspects of QoL[96]. Since pharmacological treatments have so far not been consistently efficient in the long term, they represent the last step and should be applied in the setting of clinical trials.

Screening for cachexia should ideally be carried out at the time of diagnosis of pancreatic cancer since early stages (pre-cachexia) can easily be missed although they are probably the most susceptible to any treatment intervention. Optimal screening should be performed using CT-image based techniques, since they allow for the most accurate assessment of cachexia, especially in obese patients. Since these measurements are not a standard in all CT scans today, an individual agreement with the radiologist has to be defined. In addition, nutrition risk scores and performance indexes can be used to aid decisions about form and level of treatment necessary. Monitoring of course and progress of disease should be implemented in regular intervals and should be combined with dietary counseling.

Supportive multidimensional pharmacological therapy should aim at ameliorating anemia, immunosuppression, depression and fatigue[41]. Moreover, secondary symptoms like pain, diarrhea or stomatitis need to be managed correctly to evaluate the efficacy of new treatments of cancer cachexia[97]. The evidence for interventions with resistance exercise training is not as extensive yet, but first results are promising[4,98]. Finally, the contemporary use of psychological and behavioral interventions, such as relaxation, hypnosis or group-psychotherapy, as well as careful psychosocial counseling and access to self-help groups should be provided for these terminally ill patients[12,41].

CONCLUSION

Even though a substantial amount of experimental, preclinical and clinical research has been carried out in the past 10 years, there is still no effective treatment for cancer patients suffering from cachexia. In pancreatic cancer, cachexia is encountered in up to 80% of patients and significantly contributes to the related morbidity and mortality[12].

Since many factors lead to cachexia in these patients, a multimodal treatment approach is needed, including nutritional support and pharmacological intervention as well as the treatment of symptoms exacerbating weight loss such as chronic pain, gastrointestinal disorders, fatigue and depression. Furthermore, interventions should be implemented in a stepwise manner, starting with oral nutritional support and dietary counseling from the time of diagnosis. Screening and monitoring of cachexia should be performed regularly, ideally using CT-scan based techniques.

After reviewing the current nutritional and pharmacological approaches to treat cachexia, combination protocols using anti-inflammatory, anti-oxidative nutrients and drugs seem the most promising. Treatment with single agents such as progesterones or TNF-α inhibitors has not shown to be successful and unnecessarily expose these patients to the risk of substantial adverse side effects.

New targeted therapies derived from extensive research in animal models hold promise for the future. In particular drugs targeting IL-6 and its downstream targets as well as the myostatin/ActRⅡb pathway are up-and-coming. However, it is always challenging looking into the crystal ball and new therapeutic approaches will only be available outside of clinical trials when the marketing approval will be granted. This is at least valid for new chemical entities or new indications for already existing drugs. While waiting for the results of ongoing trials, we strongly encourage further research and clinical trials on new treatments for this devastating condition. Furthermore, diagnostic criteria and design of clinical trials should be standardized as far as possible to make analyses and comparisons of future intervention trials more meaningful.

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2014 ADVANCES IN PANCREATIC CANCER

Neoadjuvant strategies for pancreatic cancer

Francesco Polistina, Giuseppe Di Natale, Giorgio Bonciarelli, Giovanni Ambrosino, Mauro Frego

Francesco Polistina, Giuseppe Di Natale, Mauro Frego, Department of General Surgery, Monselice Hospital, 35043 Monselice, Italy

Giuseppe Di Natale, Rome University "La Sapienza" School of Surgery, Piazzale Aldo Moro, 500185 Roma, Italy

Giorgio Bonciarelli, Department of Clinical Oncology, Monselice Hospital, 135043 Monselice, Italy

Giovanni Ambrosino, Department of Surgery, Malzoni Hospital, 483100 Avellino, Italy

Author contributions: Polistina F, Bonciarelli G, Frego M and Ambrosino G selected, discussed and criticized the papers for the issue; Di Natale G retrieved the articles and took part to the discussion and drawing of the manuscript; Polistina F draw the manuscript and took part to the revision process together with Frego M, Bonciarelli G and Ambrosino G.

Correspondence to: Francesco Polistina, MD, Department of General Surgery, Monselice Hospital, Ospedale di Monselice, Via G. Marconi 19, 35043 Monselice,
Italy. francescopolistina@hotmail.it

Telephone: +39-429-788272 Fax: +39-429-788080

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Abstract

Pancreatic cancer (PC) is the fourth cause of cancer death in Western countries, the only chance for long term survival is an R0 surgical resection that is feasible in about 10%-20% of all cases. Five years cumulative survival is less than 5% and rises to 25% for radically resected patients. About 40% has locally advanced in PC either borderline resectable (BRPC) or unresectable locally advanced (LAPC). Since LAPC and BRPC have been recognized as a particular form of PC neoadjuvant therapy (NT) has increasingly became a valid treatment option. The aim of NT is to reach local control of disease but, also, it is recognized to convert about 40% of LAPC patients to R0 resectability, thus providing a significant improvement of prognosis for responding patients. Once R0 resection is achieved, survival is comparable to that of early stage PCs treated by upfront surgery. Thus it is crucial to look for a proper patient selection. Neoadjuvant strategies are multiples and include neoadjuvant chemotherapy (nCT), and the association of nCT with radiotherapy (nCRT) given as either a combination of a radio sensitizing drug as gemcitabine or capecitabine or and concomitant irradiation or as upfront nCT followed by nRT associated to a radio sensitizing drug. This latter seem to be most promising as it may select patients who do not go on disease progression during initial treatment and seem to have a better prognosis. The clinical relevance of nCRT may be enhanced by the application of higher active protocols as FOLFIRINOX.

Core tip: The present paper is a review on the upcoming issue of neoadjuvant strategies for pancreatic cancer patients. Protocols, timing and results of the largest series from different strategies are here presented and discussed. To authors knowledge this is the first published paper that considers even latest papers on neoadjuvant treatment for even potentially resectable pancreatic cancer.

INTRODUCTION

Pancreatic cancer (PC) is the fourth-leading cause of death due to cancer in Western countries and accounts for nine cases per 100000 inhabitants per year in Europe[1]. The only chance for long-term survival is an R0 surgical resection that is feasible in about 10%-20% of

all cases. Cumulative survival after 5 years is less than 5% and rises to 25% for radically resected patients; in this latter group, local recurrences occur in about 50% of cases and distant metachronous metastases appear in more than 70% of patients[2,3].

Only about 20% of patients are diagnosed with surgically resectable pancreatic cancer; 40% of patients have the metastatic disease and the remaining 40% have locally advanced pancreatic cancer in either the Borderline Resectable Pancreatic Cancer (BRPC) or the Locally Advanced Pancreatic Cancer (LAPC) form[4].

DEFINITION OF LAPC AND BRPC

Due to the complexity of the anatomy in the pancreatic region, even small cancers may be found at an advanced stage where the vascular invasion is so far as to be deemed unresectable (UR). Thanks to technological progresses in the field of preoperative imaging in the last decade, a new pathological entity has arisen. BRPC is seen in a subgroup of patients in the LAPC group whose conditions are considered to be resectable with a need for vascular resection and reconstruction, but who remain at a higher risk for local recurrence. A definitive definition of BRPC is still lacking and currently there are two different classification systems: the MD Anderson Cancer Center[5] (MDACC) system and the American Hepatopancreatobiliary Association (AHPBA)/Society of Surgical Oncology (SSO)/Society for Surgery of the Alimentary Tract (SSAT) system[6], which has been endorsed by the National Comprehensive Cancer Network (NCCN) guidelines. These two classification systems substantially overlap each other with one particular difference: the abutment of the celiac trunk is classified to be "borderline resectable" in the MDACC classification while it is considered to be unresectable in the AHPBA/SSO/SSAT/NCCN classification; this discrepancy is probably due the increased confidence in pancreatic surgery associated with vascular resections of some surgical groups as compared with other surgeries[7]. The criteria for the definition of UR, LAPC and BRPC are summarized in Table 1. Moreover, the MDACC added cancer feature data from tumors and the patient's biology as considerations to create three groups of patients: (1) patients with radiologically well defined cancers; (2) patients with inconclusive but suspicious metastatic disease radiologic findings; and (3) patients with a borderline status for major abdominal surgery. This last classification may have some importance in assigning patients to a somewhat personalized treatment but, on the other hand, it further enhances the discrepancies between classification systems.

STAGING OF PANCREATIC CANCER

Triphasic, thin-cut, contrast-enhanced CT scans show an 87% success rate in diagnosing vascular invasion from pancreatic cancer[8-11]. The main limitations of this technique include its poor effectiveness of diagnosing small amounts of hepatic and/or peritoneal spread[12,13]. As a

Table 1 Definition of borderline resectable and locally advanced according to the MD Anderson Cancer Center and the American Hepatopancreatobiliary Association/Society of Surgical Oncology/Society for Surgery of the Alimentary Tract/National Comprehensive Cancer Network classification systems of stage III pancreatic cancer

Definition system	Vessel	BRPC	LAPC
MDACC	SMV	Short segment occlusion	No reconstruction feasible
	PV	Short segment occlusion	No reconstruction feasible
	SMA	Abutment	Encasement
	CHA	Abutment, short encasement	Long encasement
	CT	Abutment[1]	Encasement[1]
	Metastases	Absent	Absent
AHPBA/SSO/SSAT/NCCN	SMV	Abutment, Encasement, Occlusion	No reconstruction feasible
	PV	Abutment, Encasement, Occlusion	No reconstruction feasible
	SMA	Abutment	Encasement
	CHA	Abutment, Short encasement	Long encasement
	CT	Nor encasement or abutment[1]	Abutment[1]
	Metastases	Nor visceral nor extra-regional nodal	Nor visceral nor extra-regional nodal

[1]Differences. SMV: Superior mesenteric vein; PV: Portal vein; SMA: Superior mesenteric artery; CHA: Common hepatic artery; CT: Celiac trunk; BRPC: Borderline resectable; LAPC: Locally advanced; MDACC: MD Anderson Cancer Center; AHPBA: American Hepatopancreatobiliary Association; SSO: Society of Surgical Oncology; SSAT: Society for Surgery of the Alimentary Tract; NCCN: National Comprehensive Cancer Network.

consequence, approximately 20% of patients that were thought to be resectable on CT scans show the metastatic disease when a laparotomy is conducted[14,15].

Magnetic Resonance Imaging (MRI) is of limited interest in such a setting; this technique is effective only in cases of patients with ascites and a previously known intolerance to iodinated contrast media. Moreover MRI may be helpful in better characterizing small (< 1 cm) hepatic lesions shown by CT scans and small intrapancreatic lesions that have not yet altered the pancreatic profile[16,17].

Endoscopic ultrasounds are increasing important with an accuracy as high as 85%, with a 75% specificity and a 100% sensitivity of diagnosing pancreatic cancer resectability[18-20].

Staging laparoscopy, coupled with laparoscopic ultrasounds of the liver and pancreatic region, has shown to produce a better definition of either nodal invasion or the local invasiveness of the disease. This technique has been shown to be superior to CT scans in terms of accuracy in detecting the presence of hepatic metastases[13,21-24].

The most widely accepted criteria for selecting patients that should undergo staging laparoscopy for pancreatic cancer currently are pancreatic head tumors > 3 cm in maximum diameter, cancers located in the body and tail of the gland, and cancers with unclear margins

Table 2 Studies on chemotherapy for advanced pancreatic cancer

Ref.	CT regimen	Study	LAPC (*n*)	ORR (%)	OS median	Res rate (%)	Metastatic	ORR (%)	OS median
Conroy *et al*[33]	FOLFIRINOX *vs* Gem	Multicentric phase II trial	0	NA	NA	NA	342	31.6 *vs* 9.4	11.1 *vs* 6.4
Louvet *et al*[45]	GEMOX *vs* GEM alone	phase III	98	14.9 *vs* 27.3	10.3 *vs* 10.3	NA	215	18.3 *vs* 26.4	6.7 *vs* 8.5
Rocha Lima *et al*[46]	Irinotecan + GEM *vs* GEM alone	Multicenter, open label, phase III	51	25.9 *vs* 4.2	9.8 *vs* 11.7	NA	293	14.9 *vs* 4.8	5.4 *vs* 5.9
Poplin *et al*[47]	GEM *vs* GEM FDR *vs* GEMOX	phase II, multicentric	86	36	9.2	NA	737	NR	4.9 *vs* 6.2 *vs* 5.7
Kindler *et al*[49]	GEM + Bevacizumab *vs* GEM + placebo	Double blind, placebo controlled, phase III	31	NA	NA	NA	189	NR	5.8 *vs* 5.9
Gunturu *et al*[53]	FOLFIRINOX	Single centre, retrospective	16	50	NA	NA	19	47	NA
Peddi *et al*[55]	FOLFIRINOX	Registry	18	34	NA	NA	22	18	NA

CT: Chemotherapy; LAPC: Locally advanced pancreatic cancer; ORR: Overall response rate; OS: Overall survival; Res: Resection; NA: Not available; NR: Not reported; FOLFIRINOX: 5-fluorouracil + leucovorin + oxaliplatin + irinotecan; GEM: Gemcitabine; GEM FDR: Gemcitabine fixed dose ratio.

on imaging and Carbohydrate Antigen 19.9 > 100 U/mL in patients with normal serum bilirubin levels[25].

BASIS FOR NEOADJUVANT THERAPY

Patients with LAPC and either BRPC or UR pancreatic cancer have about a 50% chance for curative resection, as compared with stage I and II pancreatic cancers[5,26-28]. This is mainly due to the high frequency of invasion of the retroperitoneal margin and/or the nervous plexus of the SMA, whose resection adds further morbidity to the intrinsic morbidity related to pancreatic resections with vascular reconstructions[29-32]. The most significant factor predicting long-term survival in pancreatic cancer patients is an R0 resection, a widely accepted procedure. An R1 resection is associated, independent of the original stage of disease, with a prognosis similar to patients with the inoperable disease[33-42].

Early retrospective analyses showed that the chance to obtain an R0 resection for both UR-LAPC and BRPC patients was about one half compared with that for T1 and T2 patients[43-45].

At the same time, some randomized multicentric studies with adjuvant therapies for resectable pancreatic cancer showed a survival advantage for treated patients for both overall survival and disease-free survival[33,46,47], while studies performed with adjuvant radiotherapy and Intraoperative Radiotherapy showed a good efficacy in a local control of disease.

Recent meta-analyses done on early published studies on neoadjuvant treatments of BRPC and LAPC[48] showed an increased rate of R0 resections with unchanged mortality and morbidity as compared with those treated by upfront surgery. Moreover, these meta-analyses show some evidence of additional advantages over neoadjuvant strategies: (1) patients treated by upfront surgery often show a delay in the beginning of adjuvant treatment due to surgical complications, even minor ones. This fact leads to an evident survival handicap. Neoadjuvant therapy may avoid this handicap[25]. In three different studies,

patients who underwent neoadjuvant therapies completed the cycles in 90%-100% of cases, compared with 62% of patients who completed adjuvant chemotherapy in the CONLO-001 study[25]; (2) neoadjuvant therapies may help in avoiding unnecessary major abdominal surgery for patients who go on disease progression during treatment; (3) giving chemotherapic agents to a patient with pancreatic tissue not yet altered by the trauma of surgery seems to have a better effect due to better vascularization and subsequent drug delivery to neoplastic tissues[34]; (4) for BRPC and LAPC patients, neoadjuvant therapy lead to a downstaging of the disease, increasing the rate of R0 resections[5,38-44]; (5) some studies report a decreased incidence of anastomotic fistulas after neoadjuvant therapies, probably due to the pancreatic and peripancreatic fibrosis inducted by treatment[45,49-51]; and (6) two recent comparative analyses on the costs of various treatments for pancreatic cancer showed an economic advantage in neoadjuvant treatment regimens[45,52].

NEOADJUVANT CHEMOTHERAPY FOR LAPC AND BRPC

Gemcitabine-based chemotherapy

Older randomized trials exploring the effects of neoadjuvant chemotherapy alone on pancreatic cancer included both BRPC/LAPC patients and metastatic pancreatic cancer patients (Table 2). Gemcitabine-based studies evaluated both metastatic and LAPC patients and showed a 20%-30% response rate without any difference between metastatic and locally advanced. In two studies, Gemcitabine was given with Oxaliplatin *vs* Gemcitabine alone[45,46]. In one study, Gemcitabine was given with Irinotecan *vs* Gemcitabine alone[47]. In another study, Gemcitabine was given with Bevacizumab *vs* Gemcitabine alone[49]. All of these studies showed that results were significantly superior in favor of the combination therapy *vs* Gemcitabine alone but no study had resection as an endpoint. In a meta-analysis by Andriulli and others exploring the effects of Gemcitabine-based

Table 3 Studies on neoadjuvant chemotherapy for locally advanced pancreatic cancer

Ref.	Study type	CT regimen	Staging system	LAPC (*n*)	Res rate (%)	R0 resections/total resections	ORR	OS median
Lee *et al*[51]	Prospective non-randomized	Gemcitabine + capecitabine	NCCN	18 BR 25 UR	61 BR 24 UR	9/11 BR 5/6 UR	NR	23.1 mo (cumulative)
			APBCC	33 BR 10 UR	46 BR 20 UR	13/15 BR 1/2 UR	NR	
Sahora *et al*[52]	Prospective phase Ⅱ	NeoGEMTAX	NR	12 BR 13 UR	32	7/8	NR	16 mo (resected patients)
FARIS[1] *et al*[54]	Single centre, retrospective	FOLFIRINOX	NCNN	22	NR	22.7	27.3% (CT alone)	NR
Hosein[1] *et al*[39]	Prospective phase Ⅱ	FOLFIRINOX	NR	14 BR 4UR	55.5	7/8	NR	16 mo (resected patients)

[1]Some patients had CRT. ORR: Overall response rate; OS: Overall survival; NR: Not reported; NeoGEMTAX: Gemcitabine + docetaxel; FOLFIRINOX: 5-Fluorouracil + leucovorin + oxaliplatin + irinotecan; GEM: Gemcitabine; GEM FDR: Gemcitabine fixed dose ratio; NCCN: National comprehensive cancer network; APBCC: Asan pancreaticobiliary cancer center.

neoadjuvant therapy for pancreatic cancer, the reported of 1 and 2 year survival rates are 54.2% and 27% for patients with LAPC, respectively, with a complete/partial response ratio of 27% (95%CI: 18-38)[50]. A recent report by Lee *et al*[51] studied 43 patients affected by both LAPC (25) and BRPC (18), as defined by the NCCN criteria. The patients were treated with a combination of Gemcitabine and Capecitabine and the authors reported a 18.6% radiological response rate and a stable disease rate of 69.8%. In the LAPC group, 24% underwent surgical resection with 83.3% having R0 resections. In the BRPC group, 61% underwent resection with 81.8% having R0 resections. Sahora and collaborators, in a prospective study on 13 LAPC patients and 12 BRPC patients treated by neoadjuvant Gemcitabine and docetaxel chemotherapy, reported an overall resection rate of 32% with 87.5% having R0 resections and a median survival time of 16 mo for resected patients (95%CI: 8-24 mo) *vs* 12 mo for unresected patients[51]. An overview of these studies is given in Table 3.

FOLFIRINOX-based chemotherapy
Based on the work of Conroy *et al*[33] reporting on the results of a phase Ⅲ study on the efficacy of FOLFIRINOX chemotherapy on both LAPC and metastatic pancreatic cancer, this study compared FOLFIRINOX with Gemcitabine alone and showed the significant superiority of FOLFIRINOX in Overall Survival (OS), Progression Free Survival (PFR) and Overall Response Rate. Notably, as for the Gemcitabine-based studies, LAPC showed the same response rate as metastatic pancreatic cancer. Retrospective and registry analyses data on neoadjuvant FOLFIRINOX are currently available and show mostly consistent results for both metastatic pancreatic cancer and LAPC[33,53-55]. A recent retrospective study published by Hosein *et al*[39] on 18 patients with LAPC (4 BRPC and 14 UR, as defined using the AHPBA/SSO/SSAT criteria for resectability definition) report a 38.8% post-treatment radiologic resectability with a 62.5% rate of R0 resections and a 1 year progression free survival (PFS) rate as high as 83%; the 1 year overall survival rate was 100%. They did not find any statistically significant difference in survival rates between the R0 and R1 resected patients.

Moreover, several dosage adjustments of chemotherapic were required during treatment, although this procedure did not seem to interfere with the overall results[39]. A more recent study from Marthey on a preliminary prospective database with FOLFIRINOX in 53 LAPC patients showed an 83% disease control rate with a 30% response rate and a final 32% resectability rate although some of the responding patients underwent external beam radiotherapy as a continuation of the treatment schedule[56]. The preliminary result of this study is that FOLFIRINOX is effective at controlling pancreatic cancer with an overall response rate higher than 30%. This finding forecasts more focused phase Ⅲ clinical trials on this subject.

Neoadjuvant chemoradiotherapy
Evidence to support the use of neoadjuvant chemoradiotherapy (CRT) for LAPC is limited but rapidly increasing. The theoretical hypothesis that CRT is based on is that while chemotherapy provides control for a micro-disseminated disease and also acts as a radiation sensitizer, radiotherapy (RT) may have a huge impact on the local control of the disease. Since the mid-1980s, studies have been published on the treatment of LAPC by 5-FU-based CRT protocols that were shown to prolong survival when compared with radiation alone[57].

The next step was the use of Gemcitabine as a chemotherapic drug instead of 5-FU, with some evidence of a higher efficacy on both local response and overall survival[57]. In a recent meta-analysis from Gillen *et al*[40] 111 trials including as many as 4394 pancreatic cancer patients were evaluated[47]. The authors found an overall response rate of 42% for LAPC and overall disease control for 77% of LAPC patients. Laparotomies was performed in 47% of initially UR patients and, among these patients, 33% were resected; 79% had R0 resections. The overall reported median survival duration was 10.2 mo while for the 33% of resected patients, the median survival duration was 20.5 mo. Similar results were found by other systematic reviews[39,48,58-60]. A recent multi-institutional phase Ⅱ study on GEMOX-based CRT by Kim *et al*[61] clearly reports on the efficacy of their CRT protocol. This is the first prospective trial where resectability is a clear end-

point, including resectable pancreatic cancer, BRPC, and UR with a clear definition according to NCCN criteria. The study enrolled 68 patients (23 resectable, 39 BRPC and 6 UR). Sixty-six patients completed the protocol and were evaluated for surgery and 48 underwent laparotomies with 84% having R0 resections. In particular, 13 out of 19 eligible patients from the BRPC group had a post-CRT R0 resection. The median OS was 18.2 mo (95%CI: 13-26.9 mo) with the best performance for the initially resectable patients (26.5 mo). The resected patients from the BRPC pre-treatment group had a median OS of 18.4 mo.

The evidence that about 30% of LAPC patients develop systemic metastases during the early cycles of treatment[40] indicates the need for some early systemic control of the disease. To this end, some researchers treated LAPC patients with upfront chemotherapy followed by CRT. The presumed advantage of such a treatment schedule is that the early therapeutic approach may use not only RT-sensitizing drugs such as 5-FU and Gemcitabine or Capecitabine but rather drugs that are more effective against cancer. On the other hand, this approach may select patient who did not progress, thus avoiding the additional toxicity of unnecessary Radiotherapy (RT). Two retrospective and two prospective studies on this issue showed promising results. Huguet *et al*[62] used an upfront administration of Gemcitabine followed by Gemcitabine-based CRT for 71% of patients who did not progress. The authors recorded a 15 mo median OS with a 10.8 mo median PFS that was significantly better than the median OS and PFS of patients treated by Gemcitabine-based CT only in the same study. These data completely consist with those reported by Krishnan[63], who retrospectively analyzed the effect on 323 LAPC patients who had received primary CRT (247 patients) or induction Gemcitabine-based CT followed by Gemcitabine-based CRT (76 patients). They found that there was a strongly statistically significant improvement in both OS and PFS in the CT-CRT group when compared with patients in the CT-alone group (*P* < 0.001 in both cases).

Last year, data from one phase II and one phase III prospective trials on CT followed by CRT became available. Mukherjee *et al*[64] treated 114 patients (the SCALOP study) with induction Gemcitabine/Capecitabine-based chemotherapy. The 74 patients who were not progressing were randomized to undergo a course of external beam RT given with Capecitabine (36 patients) or Gemcitabine (36 patients) administered concomitantly. In the setting of a strong survival advantage for CRT patients *vs* CT alone, the authors reported a better performance in both OF and DFS for the Capecitabine group, which showed a median OS of 15.2 mo and a DFS of 12 mo *vs* 13.4 mo (OS) and 10.4 mo (DFS) for the Gemcitabine-based CRT patients (*P* < 0.001).

Leone *et al*[65] published results of upfront GEMOX CT followed by Gemcitabine-based CRT; this study is the only one to include surgery as an option while also

reporting on the resectability rate. The authors enrolled 39 patients with both BRPC (15 patients) and LAPC (24 patients) as defined by the NCCN criteria and applied an induction GEMOX-based CT and then a restaging; non-progressing patients then underwent a 50.5 Gy fractioned RT with concomitant Gemcitabine infusion on a twice-weekly base standard dose. The study reports that 94.9% of patients maintained at least a stable disease with no complete responses and 10.2% were partial responses; 15 patients were deemed resectable at the end of the treatment (38.4%) and, of these, 14 were operated on (one patient refused) with nine R0 resections (64.2%). The overall median PFS was 10.2 mo with 40% DFS at 1 year and 12% DFS at 2 years. The DFS was significantly longer for resected patients (*P* < 0.000001). The overall median OS was 16.7 mo while it was 27.8 mo for BRPC and 13.3 mo for LAPC (Table 4). These data substantially confirm that BRPC and LAPC patients converted to resectability may have a significant survival advantage and even a chance for a cure by an appropriate multimodal treatment.

NEOADJUVANT TREATMENT FOR RESECTABLE PANCREATIC CANCER

The impressive initial results obtained by neoadjuvant treatment for LAPC lead one to consider applying neoadjuvant strategies even to resectable pancreatic cancer. In a prospective phase II trial giving GEMOX CT to 28 patients, Heinrich *et al*[66] showed that neoadjuvant treatment did not affect resectability rates with a good tolerance profile since the same author began, in 2011, a randomized multicentric phase III study tht gave adjuvant Gemcitabine *vs* neoadjuvant GEMOX to resectable pancreatic cancer patients (NEOPAC study). This study is still continuing[67]. Tujima *et al*[68] reported on 34 patients with resectable pancreatic cancer randomized to receive standard upfront resection (21 patients) or two cycles of neoadjuvant therapy with Gemcitabine and oral S-1. They found no difference in resectability rates between the two groups and a statistically significant difference in the 1 and 2 years survival rates for treated patients that decreased over time to become consistent with that of the untreated patient survival rates at 3 years. More recent papers including neoadjuvant CRT are available. The study from Sho *et al*[69] compared 61 resectable (22 patients) or borderline resectable (39 patients) pancreatic cancer patients treated by a Gemcitabine-based 50 Gy fractionated course of RCT with 71 pancreatic cancer patients treated by upfront resection. The study presents some potential biases from the lack of pre-neoadjuvant Chemoradiotherapy (nCRT) histological confirmation of the diagnosis for some patients and the administration of adjuvant CT to some others. Otherwise, they report no difference in the resection rates between the groups and a statistically significant reduction in post-operative pancreatic fistulas (*P* = 0.045) and length of hospital stay (*P* = 0.0173) for nCRT patients *vs* upfront surgery. Moreover,

Table 4 Studies on neoadjuvant chemoradiotherapy for locally advanced pancreatic cancer

Ref.	Study type	CT regimen	RT	Staging System	LAPC (*n*)	Resection rate (%)	R0 resections/ total resections	ORR (%)	OS median (mo)
Shinchi *et al*[57]	Prospective randomized trial	5-FU concurrent infusion	External beam RT (50.4 Gy/28 fractions) *vs* no RT	NR	31	NR	NR	31	13.2 *vs* 6.4
Tinkl *et al*[60]	Prospective study	Gemcitabine	Three dimensional conformal 55.8 Gy tumor 50.4 Gy nodes	NR	120	31.6	35/38	NR	25
Kim *et al*[61]	Phase I study	Gemcitabine + oxaliplatin	Concurrent external beam RT 27 Gy/15 fractions	NCCN	38	28.9	7/11	15.7	12.5 (all patients)
Huguet *et al*[62]	Phase II and III trial	Upfront CT: FOLFUGEM, GEMOX, Gemcitabine *vs* GEMOX 167 patients	External beam RT (55 Gy/30 fractions) 72 patients	NR	167	NR	NR	NR	13.1
Krishnan *et al*[63]	Prospective non-randomized trial	Chemoradiation (247 patients) Upfront GEM CT followed by CRT 5-FU, GEM, CAPE (76 patients)	30 Gy (220 patients) or 55 Gy (27 patients) 30 Gy (64 patients) or 55 Gy (12 patients)	MDACC	323	NR	NR	NR	9.1
Mukerjee *et al*[64]	Open label, randomized, phase II trial	Upfront CT GEM or CAPE CRT GEM or CAPE	58 Gy/30 fractions	NR	74 38 GEM 36 CAPE	NR	NR	20.2	15.2 (GEM) *vs* 13.4 (CAPE)
Leone *et al*[65]	Prospective non-randomized trial	Upfront CT GEMOX CRT GEM	50.4 Gy	NCCN	39 15 BR 24 UR	28.2	11/11	NR	16.7 27.8 BR 13.3 UR
Polistina *et al*[76]	Prospective non-randomized trial	Upfront GEM CT GEM CRT	SBRT 30 Gy/3 fractions	MDACC	23 UR	8	2/3	69.5	10.6

NR: Not reported; RT: Radiotherapy; CT: Chemotherapy; CRT: Chemoradiotherapy; LAPC: Locally advanced pancreatic cancer; ORR: Overall response rate; OS: Overall Survival; GEM: Gemcitabine; CAPE: Capecitabine; FOLFUGEM: Leucovorin + gemcitabine + 5-fluorouracil; GEMOX: Gemcitabine + Oxaliplatin; NCCN: National comprehensive cancer network; MDACC: MD Anderson Cancer Center; BR: Borderline resectable; UR: Unresectable; SBRT: Stereotactic body radiotherapy.

there was a statistically significant reduction on nodal metastases in nCRT patients (*P* = 0.0001) and an R0 resection rate that was statistically higher in nCRT patients *vs* upfront surgery patients (92% *vs* 56%; *P* < 0.0001); no data on OS and DFS are reported.

In the most recently published study of Van Buren *et al*[70] in a phase II trial, the effects of induction fixed dose rate Gemcitabine followed by 30 Gy RT as neoadjuvant treatment of potentially resectable pancreatic cancer were examined. They enrolled 59 patients, of which 29 had BRPC. They report a 72.8% resection rate with a toxicity similar to other reported series and an R0 resection rate of 88% of resected patients (95%CI: 75-96) and a median OS of 16.8 mo (19.7 mo for resected patients).

Neoadjuvant therapy for resectable pancreatic cancer is an upcoming issue to be explored since it appears to have no significant toxicity nor shows a reduction in surgical resections. Conversely, from preliminary results, neoadjuvant therapy appears to reduce post-operative complications and hospital stay durations and also increase the rate of R0 resections. Further large randomized studies are necessary to confirm its usefulness and to assess the best treatment planning and schedule. An overview of studies is given in Table 5.

CONCLUSION

Pancreatic cancer remains a highly lethal disease in spite of all surgical, oncological, and technological progress of the last 30 years. Over this period, the prognosis of virtually all solid cancers has significantly increased; the prognosis for pancreatic cancer has remained almost the same. Pancreatic cancer patients had an overall 3% 5 year survival rate in the 1970s compared with the 5%-6% survival rate they have nowadays. In the last decade, LAPC has been recognized as an autonomous pathological entity; surgeons and oncologists have begun to try to standardize specific therapeutic strategies according to the evidence that only the achievement of surgical resection with negative margins may give a chance for a cure. The evidence that R0 resection rates are higher after neoadjuvant therapies highlights the need for research in this specific field. Based on a literature review on the issue, it appears that there are several critical points that still remain unresolved. First of all, there is a need for an univocal classification system that clearly distinguishes

Table 5 Studies on neoadjuvant chemotherapy for potentially resectable pancreatic cancer

Ref.	Study type	CT regimen	Patients (*n*)	Resection rate (%)	R0 resections rate (%)	OS median (mo)
Heinrich *et al*[66]	Prospective non-randomized phase Ⅱ	Gemcitabine + cisplatin	28	93	80	26.5
Tajima *et al*[68]	Pilot study	S1 *vs* upfront surgery	34 (total) 13 (S1) *vs* 21 (upfront surgery)	100	84.6 *vs* 85.7	2 yr 55.6% *vs* 29.6%
Sho *et al*[69]	Single centre	GEM CRT (external beam 50 to 54 Gy)	61	97	92	NR
Van Buren *et al*[70]	Prospective phase Ⅱ trial	FDR GEM + bevacizumab induction	59	72.8	38/43 (88.3%)	16. 8 (overall)
		GEM + bevacizumab Accelerated RT 30 Gy/10 fractions				19.7 (resected patients)

CT: Chemotherapy; OS: Overall survival; NR: Not reported; GEM: Gemcitabine; CRT: Chemoradiotherapy.

between borderline resectable and UR pancreatic cancer; the currently available systems do not allow for this distinction and tumors deemed "resectable" by the MDACC are deemed "unresectable" by the NCCN classification. This situation is somewhat misleading in objectively interpreting data from various groups of researchers.

Most of the studies are retrospective and done on series collected before the rise of LAPC as an independent entity, therefore the classification was done "ex-post" and this may be a further bias. Very few studies report on resectability rates or even have resection as a study end-point; the data are mostly extracted from a larger series of patients including mostly metastatic pancreatic cancer. These studies are of great significance as they show the potentialities of treatment but are non-specific and therefore potentially highly biased.

Nonetheless, an encouraging level of evidence suggests that patients undergoing neoadjuvant therapies for LAPC have a better prognosis than patients treated by upfront surgery or adjuvant therapy alone. At the same time, it appears that even patients with LAPC may have a chance for cure if down-staged to have an R0 resection, thereby achieving survival curves identical to those of primarily resectable patients. Such evidence currently suggests that even initially resectable pancreatic cancer can benefit from a neoadjuvant treatment, but this hypothesis is yet to be confirmed.

We are convinced that there is an enormous need for high-quality, randomized prospective studies that include a better selection of patients and searches for better strategies for each patient.

LOOKING FORWARD

As a future perspective, there are new advances in the field of chemotherapy agents and newer RT technologies such as Intensity Modulated Radiotherapy or Stereotactic Body Radiotherapy.

There are recently closed and ongoing trials on metastatic pancreatic cancer testing the efficacy of combined Gemcitabine and the Epidermal Growth Factor Receptor antibodies Cetuximab and Erlotinib[71,72]. However, at the present time, the results are not encouraging. Prelimi-

nary results on the use of Gemcitabine and the Vascular Endothelial Growth Factor inhibitor Axitinib did not improve outcomes in a published series[73] and the anti-HER2 drug Trastuzumab associated with Capecitabine did not seem to improve patient outcomes[74].

Recently, interest has been rising about a tumoral cytoplasmic protein involved in intracellular transport and RNA inclusion of Gemcitabine metabolites: the Intratumoral Human Equilibrative Nucleoside Transporter-1, whose presence seems to be related to responses to Gemcitabine therapy[75], but which still lacks a standard definition of a proper tissual concentration of the molecule.

Stereotactic Body Radiotherapy has been tested in some non-randomized studies. Polistina *et al*[76] treated 24 patients with intraoperatively proven UR LAPC with a 3 wk Gemcitabine CT followed by 30 Gy SBRT in three consecutive fractions and concomitant Gemcitabine at a standard dose. They report a 33% radiologic conversion to resectability and 8% R0 resections (with three patients refusing reoperation) and minimal treatment toxicity (no grade 3 or 4 events) with a 20 mo median survival time for resected patients and one histological complete tumor response. Similar toxicity and response rate results have been published by other groups[77].

SBRT CRT is a promising tool as it hypothetically adds the benefits of systemic CT to the local control of disease as obtained with more focused delivery of radiation to the tumor bed. There is less risk to nearby organs and a subsequent decreased toxicity. However, there is a strong need for prospective, randomized trials to confirm these preliminary results.

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2014 ADVANCES IN PANCREATIC CANCER

Therapeutic applications of curcumin for patients with pancreatic cancer

Masashi Kanai

Masashi Kanai, Department of Clinical Oncology and Pharmacogenomics, Graduate School of Medicine, Kyoto University Kyoto, Japan Kyoto University Hospital, Kyoto 606-8507, Japan
Author contributions: Kanai M solely contributed to this paper.
Correspondence to: Masashi Kanai, MD, PhD, Department of Clinical Oncology and Pharmacogenomics, Graduate School of Medicine, Kyoto University Kyoto, Japan Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. kanai@kuhp.kyoto-u.ac.jp
Telephone: +81-75-7514770 Fax: +81-75-7514772
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Abstract

A number of preclinical studies have demonstrated anticancer effects for curcumin in various types of tumors, including pancreatic cancer. Curcumin has anticancer effects both alone and in combination with other anticancer drugs (*e.g.*, gemcitabine, 5-fluorouracil, and oxaliplatin), and it has been shown to modulate a variety of molecular targets in preclinical models, with more than 30 molecular targets identified to date. Of these various molecules, NF-κB is thought to be one of the primary targets of curcumin activity. Based on these promising preclinical results, several research groups, including our own, have progressed to testing the anticancer effects of curcumin in clinical trials; however, the poor bioavailability of this agent has been the major challenge for its clinical application. Despite the ingestion of gram-level doses of curcumin, plasma curcumin levels remain at low (ng/mL) levels in patients, which is insufficient to yield the anticancer benefits of curcumin. This problem has been solved by the development of highly bioavailable forms of curcumin (THERACURMIN®), and higher plasma curcumin levels can now be achieved without increased toxicity in patients with pancreatic cancer. In this article, we review possible therapeutic applications of curcumin in patients with pancreatic cancer.

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Key words: Curcumin; Pancreatic cancer; Nuclear factor-kappa B; Bioavailability; THERACURMIN

Core tip: A growing body of evidence supports the idea that curcumin is a promising anticancer drug. Curcumin has anticancer effects, both alone and in combination with other anticancer drugs, through the modulation of a variety of molecular targets in preclinical models. However, the poor bioavailability of curcumin has been the major challenge to its clinical application. This problem has been overcome by the development of highly bioavailable forms of curcumin (THERACURMIN®), and higher plasma curcumin levels can now be achieved without increased toxicity. Further clinical trials will be necessary to test the therapeutic applications of this promising agent in patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies worldwide[1], and the majority of patients are diagnosed too late for curative resection. Even in patients who have undergone curative resection, the disease relapse rate within 2 years is greater than 80%[2]. Systemic gemcitabine-based chemotherapy has been a standard therapy for patients with advanced pancreatic cancer since 1997, when a randomized phase III study demonstrated that gemcitabine monotherapy significantly improved cancer-
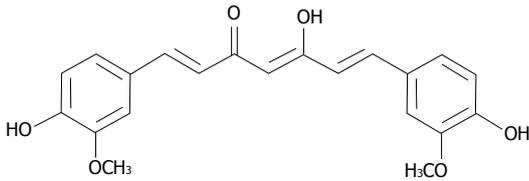
Figure 1 Chemical structure of curcumin.

related symptoms compared with 5-fluorouracil[3]. Over the past decade, many efforts have been made to improve the overall survival of patients with this disease by combining gemcitabine with a second cytotoxic agent. However, most of these gemcitabine combination therapies have failed to show significant survival advantages over gemcitabine monotherapy[4-11]. Therefore, novel approaches - other than simply adding additional cytotoxic agents to gemcitabine - are warranted. In addition, it is important to consider the balance between efficacy and quality of life when choosing a palliative chemotherapy, as patients with pancreatic cancer often suffer from cancer-related symptoms, such as fatigue, appetite loss, and pain.

Curcumin is a natural polyphenol compound derived from turmeric (*Curcuma longa*). Constituting 1%-5% of turmeric preparations, curcumin has a molecular weight of 368.37 and the molecular formula $C_{21}H_{20}O_6$ (Figure 1). Curcumin has long been used as a food (*e.g.*, in the popular Indian curry), a coloring agent and in traditional medicine[12,13]. A number of preclinical studies have demonstrated that curcumin has anticancer effects against a variety of tumors, including pancreatic cancer, both *in vitro* and *in vivo*[14-32]. These promising results have attracted the interest of many researchers hoping to develop this agent as a chemopreventive as well as a chemotherapeutic drug[33,34]. In contrast with conventional cytotoxic drugs - which often have side effects such as nausea, vomiting or fatigue - curcumin has minimal toxicity. This is a great advantage when treating patients with pancreatic cancer, who generally show poor tolerance to intensive therapy due to their poor clinical conditions. Safety is another advantage of this agent. The safety of curcumin has been approved by the Food and Drug Administration and World Health Organization; In addition, its safety is strongly supported by the fact that this agent has been used in traditional Hindu and Chinese medicine for thousands of years.

In this article, we review possible therapeutic applications of curcumin for the treatment of patients with pancreatic cancer.

ANTICANCER EFFECTS OF CURCUMIN AGAINST PANCREATIC CANCER *IN VITRO* AND *IN VIVO*

A PubMed search using the key words "curcumin" and "cancer" reveals that over 2000 articles have been pub-

lished on this topic since 1983, with that number increasing rapidly year after year. Numerous preclinical studies have demonstrated anticancer effects for curcumin against not only pancreatic cancer[14,17,22,24,26-28,32,35] but also a variety of other malignancies, including breast[21], colon[23,29], gastric[30], head and neck[25], hepatic[15], ovarian[20], lung[31] and prostate cancers[19], as well as lymphoma and leukemia[16,18].

Li *et al*[14] were the first to report the anticancer effects of curcumin against pancreatic cancer cells. They demonstrated that curcumin can suppress tumor growth in pancreatic cancer cell lines in a time- and dose-dependent manner by inhibiting nuclear transcription factor-kappa B (NF-κB). The efficacy of curcumin has also been demonstrated using an orthotopic mouse model of pancreatic cancer[36]. Although treatment with either curcumin (1 g/kg orally) or gemcitabine (25 mg/kg *via* intraperitoneal injection) had modest antitumor effects, the combination of curcumin and gemcitabine suppressed tumor growth more effectively than either agent alone. In addition to gemcitabine, curcumin has also been shown to potentiate the effects of other cytotoxic agents, including cisplatin, oxaliplatin, and 5-fluorouracil, in preclinical models[25,29,37].

Curcumin can modulate the activity of a variety of molecules that play important roles in cancer progression, with more than 30 molecular targets identified to date[38]. Of these molecules, NF-κB appears to be one of the primary targets of curcumin[14,27,36]. Interestingly, recent studies have demonstrated that changes in microRNA (miRNA) expression levels following treatment with curcumin or a curcumin analog are involved in the anticancer effects of these agents[28,39]. For example, curcumin can upregulate the expression of miR-200[28], which plays important roles in regulating the epithelial-to-mesenchymal transition (EMT) and cancer progression[40]. Conversely, curcumin can downregulate the expression of miR-21[28], which is overexpressed in a variety of tumors, including pancreatic cancer, and is considered to be an oncogenic miRNA[41]. Representative preclinical studies of the anticancer effects of curcumin against pancreatic cancer are summarized in Table 1.

Based on these promising preclinical results, several researcher groups, including our own, have progressed to testing the anticancer effects of curcumin in clinical trials.

CLINICAL TRIALS INVOLVING CURCUMIN IN PATIENTS WITH PANCREATIC CANCER

Despite numerous published preclinical studies, relatively few clinical trials have been reported so far. Several phase I and pharmacokinetic studies have been conducted using curcumin, and they found no dose-limiting toxicity (DLT) up to at least 12 g/d when administered orally to both healthy volunteers[42,43] and cancer patients[44-46]. The minor toxicities of Grade 1-2 diarrhea and nausea have been reported, although these were likely due to the

Reported molecular targets	Curcumin dose required for the reported effects	
	in vitro (μmol/L)	*in vivo*
NF-κB$\downarrow$ (Ref. 14)	$\geqslant 5.4$	NA
NF-κB$\downarrow$, cyclin-D1$\downarrow$ c-myc$\downarrow$, Bcl-2$\downarrow$ Bcl-xL$\downarrow$, cIAP-1$\downarrow$ MMP$\downarrow$, COX2$\downarrow$ VEGF$\downarrow$ (Ref. 36)	$\geqslant 25$	1 g/kg per day, *po*
NF-κB$\downarrow$, Sp-1, Sp-3, Sp4$\downarrow$ cyclin-D1$\downarrow$, survivin$\downarrow$ VEGF$\downarrow$ (Ref. 27)	$\geqslant 25$	100 mg/kg per day, intraperitoneal injection
NF-κB$\downarrow$, PGE2$\downarrow$ VEGF$\downarrow$, miR-21$\downarrow$ miR-200$\uparrow$ (Ref. 28)	$\geqslant 4$	NA

cIAP1: Cellular inhibitor of apoptosis portein-1; MMP: Matrix metalloproteinase; COX2: Cyclooxygenase-2; VEGF: Vascular endothelial growth factor; PGE2: Prostaglandin E2; NA: Not available.

ingestion of large volumes of curcumin at one time. Due to poor bioavailability, curcumin doses greater than 8 g/d do not lead to further increases in plasma curcumin levels; therefore, daily oral doses of 8 g or less have been most commonly used in clinical trials.

Dhillon *et al*[47] were the first to report a phase Ⅱ clinical trial of the effects of curcumin against pancreatic cancer. Twenty-five patients, including 3 chemo-naive patients, were enrolled in this study. Of the 22 patients that could be evaluated for responses, one patient showed a stable disease course for over 18 mo and another patient showed a partial response in a liver metastasis (73% decrease in size), although this effects lasted for only 1 month. Furthermore, curcumin treatment was found to be safe in patients with pancreatic cancer, and no toxicity was associated with curcumin intake.

Our group conducted a phase Ⅰ/Ⅱ clinical trial of curcumin in patients with pancreatic cancer who had become resistant to gemcitabine-based chemotherapy[48]. In contrast with the study by Dhillon *et al*[47], which tested the safety and efficacy of curcumin monotherapy, our study evaluated the efficacy of combined gemcitabine-based chemotherapy and curcumin treatment, which we tested based on the preclinical results showing that curcumin could potentiate the anticancer effects of gemcitabine[36]. As no previous studies had demonstrated the safety and feasibility of this drug combination in cancer patients, we began with a phase Ⅰ study involving an 8-g daily oral dose of curcumin in combination with gemcitabine-based chemotherapy. The first 3 patients that could be assessed completed their first treatment cycle without a predefined DLT. Therefore, we selected this dose for the following phase Ⅱ study. In total, 21 patients who showed disease progression during previous gemcitabine-based chemotherapy were enrolled in the study. The addition of an 8-g daily oral curcumin dose did not increase the risk of clinically relevant toxicity, and the toxicity profile of the combined drugs was comparable

with that observed in pancreatic cancer patients treated with gemcitabine-based chemotherapy alone. Cumulative toxicity from curcumin was not observed, and 4 patients were able to continue this intake regimen for over 6 mo, indicating that this agent is safe for long-term use. Even though the preliminary results were from a small sample, the observed median survival time (MST) of 5.4 (95%CI 3.6-7.4) mo and a 1-year survival rate of 19% (95%CI 4.4%-41.4%) are promising results, particularly considering the poor prognosis of patients with pancreatic cancer with resistance to gemcitabine-based chemotherapy.

Epelbaum *et al*[49] reported the results from another clinical trial testing the efficacy and feasibility of curcumin in combination with gemcitabine monotherapy in chemo-naive patients with advanced pancreatic cancer. Seventeen patients were enrolled in the study, and they received the standard dose and schedule of gemcitabine in combination with an 8-g daily oral dose of curcumin. In contrast to the previous 2 studies that showed low toxicity for 8-g daily oral doses of curcumin[47,48], this study reported that 5 patients (29%) discontinued the curcumin regimen after a period of several days to 2 wk due to intractable abdominal fullness and/or pain. Indeed, the dose of curcumin was eventually reduced to 4 g/d due abdominal complaints in 2 other patients. The researchers discussed the possibility that increased gastrointestinal toxicity could be caused by the combination of curcumin and gemcitabine, and they concluded that 8 g oral curcumin is not a viable treatment dose when combined with gemcitabine in patients with pancreatic cancer. One possible explanation for the discrepancy between our results and those of Epelbaum *et al*[49] is that the baseline clinical condition of the patients was poorer in the Epelbaum *et al*[49] study than in ours, and therefore, the abdominal fullness or pain experienced by these patients may have been primarily attributable to cancer-related symptoms.

Table 2 summarizes the published clinical trials that have tested the effects of curcumin in patients with pancreatic cancer.

APPLICATION OF A HIGHLY BIOAVAILABLE FORM OF CURCUMIN (THERACURMIN®) IN CLINICAL TRIALS

Several investigators, including ourselves, have tested plasma curcumin levels in clinical trials, and most studies have reported that plasma curcumin levels remained at low (ng/mL) levels, despite multi-gram doses of curcumin[42,45,46,48]. As described in the previous section, the intake of oral doses of curcumin greater than 8 g did not lead to further increases in plasma curcumin levels in human subjects[42-44]. Therefore, the poor bioavailability of curcumin has been the primary challenge to its clinical application. As a result, many efforts have been made to improve the bioavailability of this agent using a variety of approaches, including innovative drug delivery systems (nanoparticles, liposomes and phospholipids)[50-65] and the development of new curcumin analogs[66,67]. For

Table 2 A summary of published clinical trials testing curcumin in patients with pancreatic cancer

	Dhillon et al[47]	Kanai et al[48]	Epelbaum et al[49]	Kanai et al[69]
Sample size	25	21	17	14
Study design	Phase II	Phase I / II	Phase II	Phase I
Study period	2008[1]	2008-2009	2004-2006	2011-2012
Dose of curcumin	8 g/d	8 g/d	8 g/d	200 mg/d[2] (n = 9) 400 mg/d[2] (n = 5)
Prior history of chemotherapy	Yes (n = 22)	Yes (n = 21)	None	yes (n = 14)
Concomitant use of anticancer drug	No	Yes	Yes	Yes
Major toxicity associated with curcumin	None	None	Abdominal discomfort (n = 5)	Abdominal pain (n = 2)
Median survival time (mo)	NA	5.4	5	4.4

[1]Publication year; [2]THERACURMIN® was used in this study. NA: Not available.

Table 3 A comparison of representative studies reporting plasma curcumin levels in human subjects

	Lao et al[42]	Sharma et al[45]	Garcea et al[46]	Kanai et al[68]
Sample size	3 (1)[1]	3	3	6
Dose of curcumin (g/d)	12	3.6	3.6	0.21[1]
Plasma curcumin levels (ng/mL, mean ± SE)	57	4 ± 0.2	< 1	275 ± 67

[1]Plasma curcumin was detected in only one subject.

Figure 2 Plasma curcumin levels following administration of conventional curcumin and THERACURMIN®. Each point corresponds to an individual patient. Bars denote the median value. Adapted from Kanai et al[69].

example, a nanoparticle-based drug delivery system has been shown to improve the water solubility of hydrophobic agents such as curcumin, and several different types of nanoparticle-based curcumin have been published[52,56-59,61,62,64,65].

Of these new varieties of nanoparticle-based curcumin, we chose THERACURMIN® for further study, as it showed a greater than 30-fold increase in bioavailability compared with conventional curcumin in rat models[64]. THERACURMIN® was prepared as follows[64,68]. First, gum ghatti - which primarily consists of polysaccharides obtained from ghatti tree exudates - was dissolved in water to make a gum ghatti solution. Curcumin powder was mixed into this solution, and water and glycerin were added to adjust the final weight. This mixture was ground using a wet grinding mill (DYNO-MILL®KDL, Willy A Bachofen AG) and then dispersed with a high-pressure homogenizer (Homogenizer 15MR-8TA, APV Gaulin). Stable THERACURMIN® is obtained from this procedure.

To verify the improved bioavailability of THERACURMIN® in human subjects, we conducted a dose-escalation and pharmacokinetic study[68]. Six healthy human volunteers were recruited and given THERACURMIN® via a single oral dose of 150 mg. Following an interval of 2 wk, the same subjects were then given THERACURMIN® via a single oral dose of 210 mg. The C_{max} values for THERACURMIN® at the 150 and 210 mg doses were 189 ± 48 and 275 ± 67 ng/mL (mean ± SEM), respectively. No toxicity associated with THERACURMIN® intake was observed in this study.

These results indicate that the ingestion of THERA-

CURMIN® can lead to higher plasma curcumin levels than those achieved with conventional curcumin (Table 3). Therefore, we considered this new form of curcumin to be a promising tool for testing the potential anticancer effects of curcumin in clinical trials, and we conducted a phase I study testing the safety of THERACURMIN® in patients with pancreatic cancer[69].

A total of 16 patients (14 patients with pancreatic cancer and 2 patients with biliary tract cancer) who failed standard gemcitabine-based chemotherapy were enrolled in the study. Based on our previous pharmacokinetic study, we chose to use THERACURMIN® containing 200 mg curcumin (Level 1) as the starting dose. THERACURMIN® was administered orally every day in combination with standard gemcitabine-based chemotherapy.

Ten patients were assigned to the Level 1 group and six to the Level 2 group (THERACURMIN® containing 400 mg curcumin). Peak plasma curcumin levels (median) following THERACURMIN® administration were 324 ng/mL (range = 47-1029 ng/mL) for Level 1 and 440 ng/mL (range = 179-1380 ng/mL) for Level 2. Importantly, these values were significantly higher than the median value (85 ng/mL) observed in our previous study using 8-g doses of conventional curcumin (Figure 2). With respect to safety, two patients reported increased abdominal pain following THERACURMIN® administration. Computed tomography scans performed prior to THERACURMIN® administration in these patients revealed dilated colons, which could have been due to in-

testinal obstructions caused by peritonitis carcinomatosa.
As described in the previous section, Epelbaum *et al*[49]
reported abdominal fullness or pain following curcumin
administration in patients with pancreatic cancer. We
speculate that curcumin may irritate the intestine, poten-
tially increasing abdominal pain in patients with intestinal
obstructions due to peritonitis carcinomatosa or other
complications. In future clinical trials, we advise caution
when administering curcumin to these types of patients.

Other observed toxicities were comparable to those
for gemcitabine-based chemotherapy alone, and repeti-
tive exposure to high concentrations of curcumin did
not cause any unexpected serious adverse events, nor
did they increase the incidence of adverse events in pa-
tients with pancreatic cancer receiving gemcitabine-based
chemotherapy. In fact, three patients safely continued
THERACURMIN® treatment for > 9 mo. With respect
to efficacy, no responses were observed in this study
based on RECIST; however, the MST was 4.4 mo (95%
confidence interval: 1.8-7.0 mo) for the 14 patients with
pancreatic cancer, and three patients (21%) survived for
> 12 mo following initiation of THERACURMIN®.

Interestingly, fatigue- and functioning-associated qual-
ity of life (QOL) scores scaled by EORTC QLQ-C30
significantly improved following THERACURMIN® ad-
ministration. In five patients, the fatigue score improved
by > 20, which was interpreted as a significant and clini-
cally relevant change[70]. Preclinical and clinical studies
demonstrating the benefits of curcumin on heart disease,
depression, and fatigue, also support these findings[71-73].
As improved QOL has been demonstrated to contribute
to better outcomes in cancer patients[74], it is tempting to
speculate that THERACURMIN® may prolong the over-
all survival of patients with pancreatic cancer through
QOL improvements. A randomized placebo-controlled
clinical trial is now underway to verify this hypothesis
(UMIN000010326).

CONCLUSION

A growing body of evidence supports the idea that cur-
cumin is a promising anticancer drug. In preclinical mod-
els, curcumin has been shown to have anticancer effects,
both alone and in combination with other anticancer
drugs, through the modulation of a variety of molecular
targets. However, the poor bioavailability of curcumin
has been the major challenge to its clinical application.
This problem has now been solved by the development
of highly bioavailable forms of curcumin (THERA-
CURMIN®), which can induce higher plasma curcumin
levels without increased toxicity. Further clinical trials will
be necessary to test the therapeutic applications of this
promising agent in patients with pancreatic cancer.

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Pathophysiological roles of Pim-3 kinase in pancreatic cancer development and progression

Ying-Yi Li, Naofumi Mukaida

Ying-Yi Li, Cancer Research Institute, Fudan University Shanghai Cancer Center, Shanghai 200433, China

Ying-Yi Li, Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200433, China

Ying-Yi Li, Division of Molecular Bioregulation, Cancer Microenvironment Research Program, Cancer Research Institute, Kanazawa University, Kanazawa 920-1192, Japan

Naofumi Mukaida, Division of Molecular Bioregulation, Cancer Microenvironment Research Program, Cancer Research Institute, Kanazawa University, Kanazawa 920-1192, Japan

Correspondence to: Naofumi Mukaida, MD, PhD, Professor, Division of Molecular Bioregulation, Cancer Microenvironment Research Program, Cancer Research Institute, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan. mukaida@staff.kanazawa-u.ac.jp

Telephone: +81-76-2646735 Fax: +81-76-2344520

Abstract

Pim-3 is a member of the provirus integration site for Moloney murine leukemia virus (Pim) family proteins that exhibit serine/threonine kinase activity. Similar to the other Pim kinases (Pim-1 and Pim-2), Pim-3 is involved in many cellular processes, including cell proliferation, survival, and protein synthesis. Although Pim-3 is expressed in normal vital organs, it is overexpressed particularly in tumor tissues of endoderm-derived organs, including the liver, pancreas, and colon. Silencing of Pim-3 expression can retard *in vitro* cell proliferation of hepatocellular, pancreatic, and colon carcinoma cell lines by promoting cell apoptosis. Pim-3 lacks the regulatory domains similarly as Pim-1 and Pim-2 lack, and therefore, Pim-3 can exhibit its kinase activity once it is expressed. Pim-3 expression is regulated at transcriptional and post-transcriptional levels by transcription factors (*e.g.*, Ets-1) and post-translational modifiers (*e.g.*, translationally-controlled tumor protein), respectively. Pim-3 could promote growth and angiogenesis of human pancreatic cancer cells *in vivo* in an orthotopic nude mouse model. Furthermore, a Pim-3 kinase inhibitor inhibited cell proliferation when human pancreatic cancer cells were injected into nude mice, without inducing any major adverse effects. Thus, Pim-3 kinase may serve as a novel molecular target for developing targeting drugs against pancreatic and other types of cancer.

Core tip: The present review describes the current knowledge on the roles of Pim-3 in pancreatic cancer development and progression, and provides the possibility for Pim-3 as a therapeutic target in human pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States[1]. Patients usually

suffer from non-specific abdominal discomfort in the primary stages, which often delay early diagnosis and treatment. Furthermore, even in the initial evolutionary phase of disease development, pancreatic cancer cells tend to undergo invasion and metastasis. Therefore, complete removal of tumors by surgical procedures is often impossible. Another major stumbling block in treating pancreatic cancer is its frequent resistance to chemotherapy and radiotherapy treatment. Consequently, pancreatic cancer has an exceptionally poor prognosis, with an overall 5-year survival rate of less than 5%[2,3]. Thus, a novel molecular targeted therapy will be a required therapeutic option for human pancreatic cancer treatment.

Malignant lesions of the pancreas show a ductal, acinar, or endocrine lineage. Nearly 80% of pancreatic carcinomas are classified as pancreatic ductal adenocarcinoma (PDAC)[4]. An activating mutation in a key proto-oncogene has been observed in most PDACs, and is presumed to be the first significant event involved in pancreatic carcinogenesis[4]. The development and progression of PDAC is associated with additional multiple genetic and epigenetic alterations in several proto-oncogenes, tumor-suppressor genes, and signaling pathways. Pim-3 kinase has essential roles in the regulation of signal transduction cascades. Moreover, its expression is enhanced in human pancreatic cancer cell lines, and blocking of its expression induced apoptosis and decreased chemoresistance in human pancreatic cancer[5,6].

The provirus integration site for the Moloney murine leukemia virus (Pim) family is a proto-oncogene, which belongs to the calcium/calmodulin-regulated kinase group and exhibits serine/threonine kinase activity[7]. The Pim family consists of three members: Pim-1, Pim-2, and Pim-3[8]. The *Pim-1* gene was first discovered as a proviral insertion site in Moloney murine leukemia virus[9]. A subsequent study demonstrated that Pim-1 transgenic mice are predisposed to the development of experimental T cell lymphoma in cooperation with c-Myc and N-Myc[10]. Pim-2 was similarly identified as a proviral integration site in Moloney murine leukemia virus-induced T cell lymphomas[11], and can synergize with c-Myc-induced lymphomagenesis[8]. Pim-3 was first identified as a novel gene induced by membrane depolarization or forskolin in rat PC12 pheochromocytoma cells, and was designated as kinase induced by depolarization (KID-1)[12]. Subsequently, KID-1 was renamed Pim-3 due to its high sequence similarity with the other Pim family proteins Pim-1 and Pim-2. Although Pim-3 can be detected in several normal tissues, including those of the brain and heart, it is expressed in high levels in tumor tissues of various organs, particularly those of endoderm-derived organs such as the pancreas, liver, colon, and stomach[5,13,14].

In this review, we aim to highlight the pathophysiological roles of Pim-3 in the development and progression of cancer, particularly pancreatic cancer. Moreover, by considering the sequence similarity of Pim-3 with other Pim kinases, we were able to rationalize and predict the possible functions of Pim-3 by extrapolating from the data established for other Pim family members, particularly Pim-1. We further discuss the potential of Pim-3 as a novel molecular target for antineoplastic therapy.

STRUCTURE OF PIM-3 PROTEIN

The open reading frame of human Pim-3 mRNA encodes a protein consisting of 326 amino acids with a calculated molecular weight of 35861 (Figure 1)[13]. Human Pim-3 protein shares a high percentage of sequence homology with other members of the Pim family; Pim-3 and Pim-1 are 71% identical at the amino acid level, and Pim-3 and Pim-2 are 44.0% identical[14-17].

The crystal structure of the Pim-3 protein has not yet been established, but several research groups have independently reported the crystal structure of Pim-1 and Pim-2 in the free form, as well as in complex with their inhibitors[18-22]. The Pim-1 kinase adopts a two-lobe kinase fold connected by a hinge region (residues 121-126)[18]. The N-terminal lobe is composed of anti-parallel β-sheets, while the C-terminal lobe is composed mainly of α-helices (Figure 1). The adenosine triphosphate (ATP)-binding site is located in a deep intervening cleft between the two lobes and the hinge region. The Pim family proteins have no regulatory domains. Moreover, the ATP binding pocket in Pim-1 remains open irrespective of the presence or absence of ATP[18], indicating a continuous maintenance of an active state conformation. Similar findings have been reported for the structure of Pim-2 kinase[20]. This may account for the good correlation between protein expression levels and overall kinase activity in the case of Pim-1 and Pim-2[15]. Given the high sequence similarity (Figure 1 and NCBI Reference Sequence: NP_001001852.2), it is highly likely that Pim-3 kinase can adopt a similar three dimensional active conformation. Importantly, several residues believed to confer specificity in Pim-1 kinase are also conserved within Pim-2 and Pim-3 proteins.

MECHANISMS UNDERLYING CONTROL OF PIM-3 EXPRESSION

Pim-3 mRNA is detected in several normal human tissues, including the heart, brain, lung, kidney, spleen, placenta, skeletal muscle, and peripheral blood leukocytes, but not in the colon, thymus, liver, or small intestine[13]. Pim-3 is expressed in endothelial cells[23]. Focal cerebral ischemia enhances Pim-3 mRNA expression in the peri-infarction cortex at early time points[24]. Similarly, ischemia reperfusion injury enhances intra-cardiac *Pim-3* expression through the p38-mediated signaling pathway[25]. In the mouse embryo, *Pim-3* gene expression is detected in the liver, kidneys, lungs, thymus, central nervous sys-
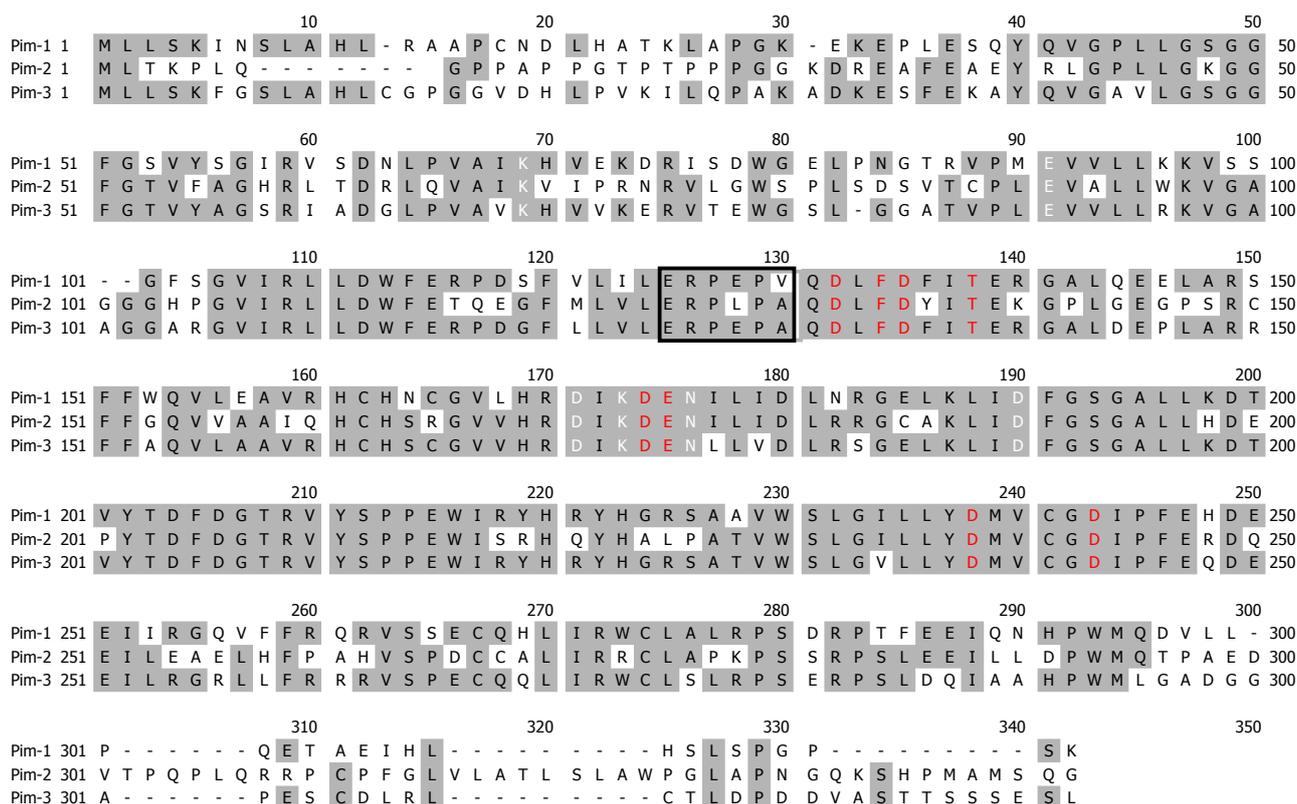
Figure 1 Amino acid alignment of human Pim family proteins[13]. The amino acid sequences of human Pim family kinases are aligned and common residues shared with Pim-3 are highlighted. The box indicates the hinge region. Residues marked with white and red are important for adenosine triphosphate binding and substrate selectivity, respectively.

tem, periphery of the pancreas, secretory epithelium of the stomach, and intestinal epithelium[26].

Pim-3 mRNA is found to be expressed in a panel of human Ewing's family tumor cell lines[27] and naso-pharyngeal carcinoma cell lines[28]. Likewise, we revealed that Pim-3 protein is scarcely detected in adult normal endoderm-derived organs, such as the liver, pancreas, colon, and stomach, but its expression is augmented in premalignant and malignant lesions of these organs (Table 1)[5,13,29,30]. Pim-3 protein is mostly detected in the cytoplasm of these tumors. In the liver, aberrant expression of Pim-3 protein is also observed in precancerous lesions such as regenerative nodules and adenomatous hyperplasia[13]. Similarly, in the colon and stomach, Pim-3 protein is detected at higher levels in adenoma tissues compared with adenocarcinoma tissues[29,30]. These observations suggest that Pim-3 plays a crucial role in the initial phase of carcinogenesis.

Pim-3 expression is regulated mainly at transcriptional and post-transcriptional levels. We will discuss the regulatory mechanisms of these two levels in detail.

Transcriptional regulation of Pim-3 expression

Ewing's sarcoma (EWS)./Ets fusion proteins are pathognomonic for EWS. These fusion proteins arise from the chromosomal translocations that combine a portion of the amino-terminal region of EWS to one of the five

members of Ets family transcription factors: friend leukemia integration transcription factor (FLI), Ets-related gene (ERG), FEV, Ets translocation variant 1, or Ets translocation variant 4[31]. Deneen *et al*[27] demonstrated that EWS/Ets fusion proteins can enhance *Pim-3* gene transcription in NIH 3T3 cells.

We have determined the 5'-flanking region of the human *Pim-3* gene as that necessary in order to elucidate the molecular mechanisms underlying constitutive Pim-3 expression in human pancreatic cancer cells. The human *Pim-3* gene contains a canonical TATA box and putative binding sites for several known transcription factors, such as signal transducer and activator of transcription (Stat)3, Sp1, and Ets-1, as well as nuclear factors NF-κB and NF-1[32]. Pim-3 expression is enhanced in murine embryonic stem cells by leukemia inhibitory factor (LIF)/gp130-dependent signaling and the Stat3 transcription factor[33]. In contrast, the transfection of the dominant negative form of Stat3 failed to inhibit the promoter activity of the *Pim-3* gene in human pancreatic cancer cells[32]. We further demonstrated that the region between -264 and -164 bp is essential for constitutive *Pim-3* gene expression. This region contains one NF-κB, two Sp1, and two Ets-1 binding sites. *Pim-1* gene can be induced by CD40-mediated signaling in an NF-κB-dependent manner[34]. However, mutation in the NF-κB binding site of the *Pim-3* gene failed to reduce promoter

Table 1 Increased expression patterns for Pim kinases in various types of malignancies

Tumor subtype	Pim-1	Pim-2	Pim-3
Solid tumor			
Pancreatic cancer	+	ND	+
Colon carcinoma	+	ND	+
Gastric cancer	+	ND	+
Hepatocellular carcinoma	+	+	+
Prostate adenocarcinoma	+	+	+
Bladder carcinoma	+	ND	ND
Squamous cell carcinoma of head and neck	+	ND	ND
Nasopharyngeal carcinoma	ND	ND	+
Oral squamous cell carcinoma	+	ND	ND
Liposarcoma	+	ND	ND
Ewing's sarcoma	ND	ND	+
Hematological malignancies			
Acute myeloid leukemia	ND	+	ND
B Cell chronic lymphocytic leukemia	ND	+	ND
Primary mediastinal large B cell lymphoma	+	ND	ND
Mantle cell lymphoma	+	+	ND
Diffuse large B cell lymphoma	+	+	ND
Burkitt's lymphoma	+	ND	ND

ND: Not determined.

activities in human pancreatic cancer cells[32]. Further examination has revealed that the two Sp1 binding sites and the distal Ets binding site are crucial for constitutive *Pim-3* gene expression in human pancreatic cancer cells. The crucial roles of Ets-1 in constitutive *Pim-3* gene expression are further supported by our observations that the overexpression of Ets-1 enhances *Pim-3* expression, whereas the transfection of the dominant negative form of Ets-1 or Ets-1 small interfering RNA (siRNA) reduces *Pim-3* expression in human pancreatic cancer cells[32]. As the expression of both Ets-1 and Sp1 is enhanced in various types of cancer, including pancreatic cancer[35,36], Ets-1 and Sp1 may act cooperatively to induce constitutive *Pim-3* gene expression as observed with their other target genes[37].

Post-transcriptional regulation of Pim-3 expression

Pim kinase mRNAs have multiple copies of AUUUA sequences in their 3' untranslated regions (UTR); a typical characteristic sequence of mRNA with a short half-life. GC-rich sequences are present in the 5' UTR of Pim mRNAs and frequently require cap-dependent translation. Indeed, the overexpression of eukaryotic translation initiation factor 4E (eIF4E) leads to an increase in Pim-1 protein levels, indicating that Pim-1 mRNA is translated in a cap-dependent manner[38]. Moreover, the eukaryotic translation initiation factor eIF4E can bind a stem-loop-pair sequence present in the 3' UTR of Pim-1 mRNA, which allows nuclear export and translation of Pim-1 transcript[39]. Since Pim-3 mRNA shows analogous sequences as Pim-1 mRNA, the translation of Pim-3 mRNA can be regulated in a similar way.

Similar to Pim-1, Pim-3 can autophosphorylate some of its serine residues, but whether this has any functional significance is yet to be determined[19]. Moreover, Pim-1

and Pim-3 have been shown to bind to the serine/threonine protein phosphatase 2A (PP2A), resulting in their dephosphorylation, ubiquitination, and proteasomal degradation[40,41].

3'UTR of Pim-1 harbors multiple binding sites for miRNAs, including miRNA-33[42], miRNA-16[43], miRNA-1[44], miRNA-328[45], and miRNA-210[46]. miRNAs are generally highly conserved evolutionarily[42]. They can bind to the putative target sites present in the 3'UTR of the *Pim-1* gene and directly inhibit its expression at the post-transcriptional level, thereby blocking proliferation and growth of cancer and smooth muscle cells. The relevant analysis for the structure of human *Pim-3* mRNA indicates that the 3'UTR of the *Pim-3* gene harbors multiple binding sites for miRNAs (www.ebi.ac.uk; www.microrna.org). It will be interesting to know whether Pim-3 translation can be regulated in a similar manner.

We have identified a translationally controlled tumor protein (TCTP/TPT1) that interacts with Pim-3 by using yeast two-hybrid screening[47]. TCTP was aberrantly expressed and co-localized with Pim-3 in human pancreatic cancer cells. Mapping studies have confirmed that this co-localization is due to the interaction between the amino acids in the C-terminal fold of Pim-3 and the amino acids in the N-terminal sequence of TCTP. Pim-3 had no effect on TCTP expression or phosphorylation, although overexpression of TCTP increased Pim-3 expression in a dose-dependent manner. RNAi-mediated ablation of TCTP expression reduced Pim-3 protein, but not mRNA *via* the ubiquitin-proteasome degradation pathway. The resultant reduced *Pim-3* expression eventually inhibited tumor growth *in vitro* and *in vivo* by arresting cell cycle progression and enhancing apoptosis. Furthermore, *TCTP* and *Pim-3* expression were significantly correlated in pancreatic adenocarcinoma specimens and in tumors from patients showing high expression levels of *TCTP* and *Pim-3* obtained at an advanced stage of cancer. Thus, TCTP-mediated enhancement of Pim-3 protein expression may be involved in the regulation of cell cycle progression and apoptosis in pancreatic carcinogenesis[47].

BIOLOGICAL FUNCTIONS OF PIM-3

Treatment with Pim-3 shRNA can decrease *in vitro* proliferation of various types of cancer cells by inducing apoptosis[5,13,29]. The major function of BAD (Bcl-2-associated death promoter), a pro-apoptotic BH3-only protein, is to regulate apoptosis. Unphosphorylated BAD binds and eventually inactivates anti-apoptotic family members, primarily Bcl-X$_L$, but also Bcl-2. Phosphorylation of BAD at Ser^{112}, Ser^{136}, and Ser^{155} impairs its binding to Bcl-X$_L$ and Bcl-2, and the translocation of BAD from the surface of mitochondria to the cytosol is guided by the protein 14-3-3. The presence of unbound Bcl-X$_L$ maintains mitochondrial membrane potential and inhibits apoptosis[48,49]. Pim-1 and Pim-2 can phosphorylate BAD at Ser^{112}, while Akt phosphorylates Ser^{136} and
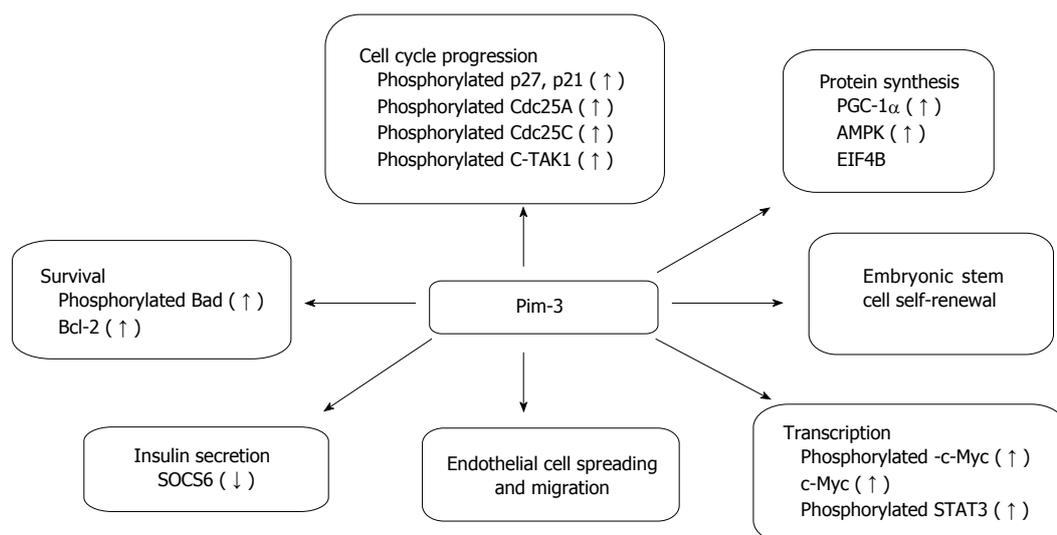
Figure 2 Presumed biological functions of Pim-3. Pim-3 can interact with various target molecules, and thereby regulates various biological pathways including apoptosis, cell cycle, protein synthesis, and transcription.

Ser[155]. The phosphorylation of BAD can result in its inactivation, leading to the subsequent inhibition of apoptosis[50,51]. Elevated levels of Pim-3 increases the amount of BAD phosphorylated at Ser[112] and inhibits apoptosis, while Pim-3 shRNA treatment dephosphorylates Ser[112] and promotes apoptosis (Figure 2)[5,29]. Therefore, similar to Akt and other Pim kinases, Pim-3 can modulate apoptosis by phosphorylating the pro-apoptotic molecule, BAD. Moreover, *Pim-3* gene transduction increased Bcl-2 expression, suppressed apoptosis (as evidenced by reduced caspase-3 activation), and eventually protected against hepatic failure in D-galactosamine-sensitized rats receiving lipopolysaccharide[52]. Similarly, the transfection of the *Pim-3* gene into cardiomyocytes attenuated ischemia/reperfusion injury-induced cell death through a p38 mediated MAPK signaling pathway[25]. Erythropoietin can protect renal cells from apoptosis by activating Stat5, and this anti-apoptotic effect is also mediated by Pim-3[53].

Pim-3 shows a high sequence identity with Pim-1, even at their kinase domains (Figure 1). Both Pim-1 and Pim-3 bind to a consensus peptide substrate (AKRRRRHPSGPPTA) with a remarkable high affinity (Kd = 40-60 nmol/L), whereas the binding affinity of this peptide for Pim-2 is relatively weak (640 nmol/L)[19]. Therefore, Pim-1 and Pim-3 can phosphorylate the same or a similar set of substrates, and the evaluation of Pim-1 characteristics and functions can provide useful insights into deciphering the major biological functions of Pim-3. In addition to BAD, Pim kinases can phosphorylate a wide range of cellular proteins. These include transcription factors (Stat[54], c-Myc[55], Myb[56], and runt-related transcription factors RUNX1 and RUNX3), cell cycle regulators (p[21CIP], p[27KIP1], Cdc25A, and Cdc25C), signaling pathway intermediates (suppressor of cytokine signaling 1 (SOCS1)[57], SOCS3[58], and MAP3K5[59]), and

regulators of protein synthesis (eukaryotic translation initiation factor 4B (eIF4B))[60].

Pim-1 can phosphorylate Cdc25A, thereby increasing its phosphatase activity and the activity of cyclin D1-associated kinases, which can result in cell cycle progression[61]. Pim-1 phosphorylates Cdc25C-associated kinase 1 (C-TAK1), which can potently inhibit Cdc25C and promote cell cycle progression at the G2/M phase[62]. Pim-1 can phosphorylate the threonine residue of p21, another molecule involved in cell cycle progression. Its phosphorylation leads to its relocation to the cytoplasm, resulting in enhanced protein stability and eventually leading to increased cell proliferation[63,64]. All Pim kinases, including Pim-3, can phosphorylate CDK inhibitor p27 at its threonine residues, thereby inducing the binding of p27 to 14-3-3 protein, resulting in its nuclear export and proteasome-dependent degradation[65]. Moreover, Pim-1 phosphorylates and inactivates forkhead transcription factors FoxO1a and FoxO3a, resulting in depressed *p27* gene transcription, which leads to cell cycle progression (Figure 2)[65]. Similarly, transfection with Pim-3 shRNA reduced the G1 population of human pancreatic cancer cells compared with the cells transfected with scramble shRNA[5]. Moreover, a small-molecule Pim-3 kinase inhibitor markedly retarded *in vitro* growth of human pancreatic cancer cell lines by inducing G2/M arrest[66], suggesting a potential role for Pim-3 in cell cycle progression. Cell cycle progression is consistently accelerated in hepatocytes of transgenic mice, which express human Pim-3 cDNA selectively in hepatocytes[67], and downregulation of Pim-3 decreased the amounts of Cdc25C, cyclin B1, and phospho-p21 (our unpublished data). Thus, Pim-3 can promote cell cycle progression and eventually contribute to carcinogenesis by modulating the functions of these regulatory molecules involved in cell cycle progression.

Mice deficient in all three Pim kinases are designated as triple knockout (TKO) mice. TKO mice have reduced body size at birth and throughout the postnatal period of their life, but are viable and fertile[68]. However, TKO mouse-derived embryonic fibroblasts (MEFs) show depressed AMP-dependent protein kinase (AMPK) activity, grow slowly in culture medium, and have decreased rates of 5'-cap-dependent protein synthesis[69]. Transduction of the *Pim-3* gene alone into these MEFs can reverse AMPK activation, increase protein synthesis, and drive the growth to a similar level as wild-type MEFs. Moreover, Pim-3 expression can markedly increase the levels of c-Myc and the peroxisome proliferation-activated receptor γ co-activator 1α (PGC-1α), enzymes capable of regulating glycolysis, and mitochondrial biogenesis[69]. Similarly, Pim-1 and Pim-2 phosphorylate serine and threonine residues of c-Myc protein[55]. Furthermore, Pim-1 can act as a co-activator of Myc by phosphorylating Ser^{10} of histone H3 on the nucleosome at the Myc-binding sites[70]. Thus, Pim-3 can augment the rate of protein synthesis by modulating AMPK, c-Myc, and PGC-1α (Figure 2).

Pim-1 and Pim-3 together play a significant role in maintaining the self-renewal capacity of mouse embryonic stem (ES) cells *in vitro*[33]. ES cells overexpressing Pim-1 and Pim-3 have a greater capacity to self-renew and display a greater resistance to LIF deprivation, as evidenced by a clonal assay. On the other hand, ablation of *Pim-1* and *Pim-3* genes increases the rate of spontaneous differentiation in a self-renewal assay, and impairs the growth of undifferentiated ES cell colonies with increased rate of apoptosis[33].

Pim-3 is highly expressed at the cellular lamellipodia in endothelial cells, and is co-localized with focal adhesion kinase (FAK). In addition, Pim-3 shRNA treatment impairs endothelial cell spreading, migration, and proliferation, leading to a reduction in tube-like structure development in a Matrigel assay[23]. However, TKO mice did not display any apparent abnormal phenotypes in embryogenesis or vascular development[68].

Pim-3 expression is detected in the β cells located in the pancreatic islets[71]. Pim-3-deficient mice exhibit an increased glucose tolerance and insulin sensitivity. Moreover, Pim-3 can negatively regulate insulin secretion by inhibiting the activation of Erk1/2 *via* SOCS6[71]. In contrast, the inhibition of another survival kinase, Akt, can induce hyperglycemia[72,73].

The switch from the latent phase to productive viral reactivation (lytic phase) is crucial for sustaining viral multiplication in infected host cells. Findings from a recent clinico-epidemiological study indicated the importance of lytic reactivation in the development and progression of Kaposi's sarcoma (KS)[74]. Latency-associated nuclear antigen (LANA) is presumed to be a novel regulator of the life cycle of γ herpes virus, including Kaposi's sarcoma herpes virus (KSHV). Pim-1 and Pim-3 contribute to the viral reactivation of KSHV by phosphorylating LANA, and thereby promote KS progression[74].

ROLES OF PIM-3 IN CANCER DEVELOPMENT AND PROGRESSION, PARTICULARLY IN THE PANCREAS

Pim-3 can contribute to cancer development and progression by acting on tumor cells and microenvironments. The primary activities of Pim-3 on tumor cells include the delivery of survival signaling, the regulation of cell cycle progression, protein synthesis, and Myc activation (Figure 3). In addition to its effects on tumor cells, Pim-3 can have profound impacts on tumor microenvironments, especially the neovascularization process (Figure 3). In the following sections we will discuss the roles of Pim-3 in carcinogenesis, with a focus on these two aspects.

Effects of Pim-3 on tumor cells

Forced expression of *Pim-3* can promote anchorage-independent growth, whereas co-expression of a kinase-dead *Pim-3* mutant can attenuate EWS/FLI-mediated NIH 3T3 tumorigenesis in immunodeficient mice[27]. These observations suggest the involvement of Pim-3 in cancer development and progression.

Pim-3 can prevent apoptosis in pancreatic cancer cells by phosphorylating BAD, a pro-apoptotic molecule on the serine residues (Ser^{112}, Ser^{136}, or Ser^{155}), which in turn prevents Bcl-X$_L$ binding and promotes BAD translocation from the surface of the mitochondria to the cytosol *via* the protein 14-3-3[48,49]. Among the serine residues present in BAD, Ser^{112}, but not Ser^{136} and Ser^{155}, is abundantly phosphorylated in human pancreatic cancer cell lines. Moreover, the ablation of endogenous Pim-3 reduces the population of phosphorylated BAD, followed by an enhancement of apoptosis, whereas Pim-3 overexpression produces exactly the opposite phenotypes. These observations suggest that Pim-3 has a crucial role in preventing apoptosis of human pancreatic cancer cells.

Cell survival can be regulated by Wnt/β-catenin and Stat3 signaling pathways. An integrative molecular screening using siRNA identified Pim-3 as a new regulator of Wnt/β-catenin signaling[75]. Thus, Pim-3 can positively regulate the Wnt/β-catenin signaling pathway in the colorectal cancer cell lines (DLD-1 and SW480)[75]. Moreover, Pim-3 is a positive regulator of Stat3 signaling in the prostate cancer cell line (DU-145) and in the pancreatic cancer derived cell line (MiaPaCa2)[56]. Thus, Pim-3 can promote cancer cell survival by modulating Wnt/β-catenin and/or Stat3 signaling pathways.

Pm-1 can promote cell cycle progression by phosphorylating and modulating the functions of molecules involved in cell cycle progression. Moreover, Pim kinases positively regulate transcription factors controlling the expression of genes implicated in cell cycle progression[65]. Since Pim-3 shares a high sequence identity with Pim-1, it is possible that Pim-3 can perform similar regulatory functions as Pim-1. Treatment with Pim-3 shRNA
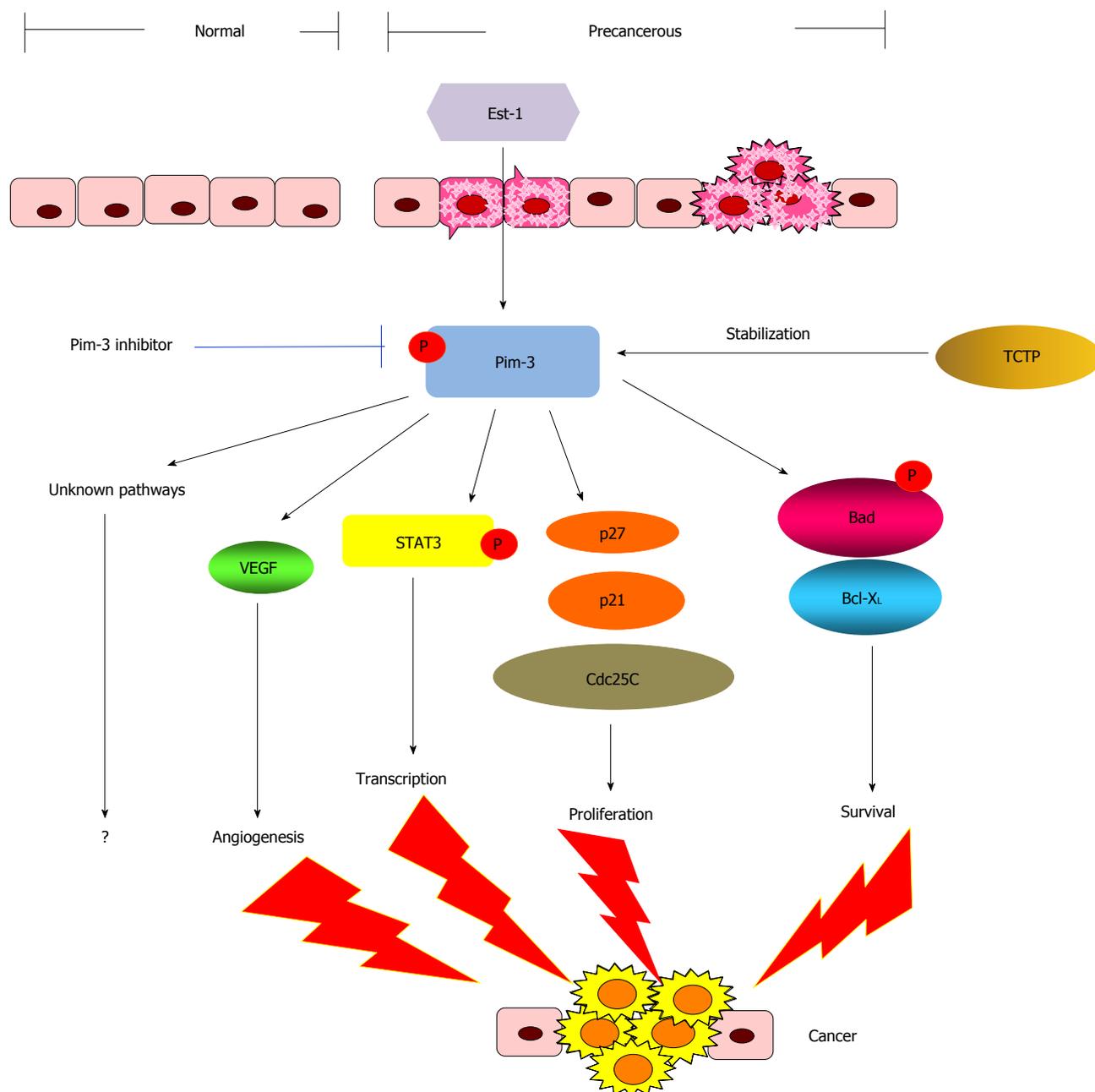
Figure 3 Presumed roles of Pim-3 in pancreatic carcinogenesis. Pim-3 expression is regulated at transcriptional and post-transcriptional levels by transcription factors (such as Ets-1) and post-translational controllers (such as translationally controlled tumor protein), respectively. Pim-3 kinase activation contributes to pancreatic carcinogenesis by inducing cell survival, cell cycle progression, gene transcription, protein synthesis in tumor cells, and angiogenesis.

showed a marked reduction in the G1 population of human pancreatic cancer cells, while scramble shRNA had few effects[5]. Furthermore, a small-molecule Pim-3 kinase inhibitor markedly retarded the *in vitro* growth of human pancreatic cancer cell lines by inducing G2/M arrest[66]. These findings indicate that Pim-3 may have a major influence in cell cycle progression of cancer cells.

Pim-1 and Pim-2 help in cell survival by suppressing myc-induced apoptosis[10,11]. Transgenic mice expressing *Eµ* (immunoglobulin heavy-chain enhancer)-*Pim-1* and *Eµ-Myc* succumb to lymphoma in utero or around birth[76]. On the contrary, *Eµ-Myc* transgenic mice that are deficient in *Pim-1* and *Pim-2* genes develop lym-

phoma slowly over time[8]. Thus, Myc-driven tumorigenesis depends on physiological levels of *Pim-1* and *Pim-2* expression. Several mechanisms have been proposed to explain the cooperation between Myc and Pim kinases. Myc recruits Pim-1 to the E-boxes of the Myc target genes such as Fos-related antigen 1 [*FOSL1* (*Fra-1*)], DNA-binding protein inhibitor *ID2*, and Pim-1 phosphorylates Ser[10] of histone H3 on the nucleosome at the Myc-binding sites that acts as a co-activator of Myc[70]. An expression profile analysis demonstrated that about 20% of Myc-regulated genes are also under the control of Pim-1[70]. Moreover, Pim-1 and Pim-2 phosphorylate c-Myc protein at its serine and threonine residues[55].

This results in the stabilization and subsequent enhancement of the transcription activities of c-Myc protein. Furthermore, Pim-3 can enhance c-Myc mRNA expression through the activation of PGC-1α[69]. The enhanced expression of c-Myc and PGC-1α may account for enhanced glycolysis. Thus, Pim kinases can promote tumorigenesis by modulating the activities of c-Myc and promoting Warburg effects[10,11].

Roles of Pim-3 in tumor microenvironments

One of the basic characteristic features of tumor tissues is the abundance of newly formed vasculature for the supply of nutrients and oxygen to the growing tumor cells, as well as the elimination of metabolic wastes and carbon dioxide. Pim-3 is abundantly expressed at mRNA and protein levels at the cellular lamellipodia, and is co-localized with FAK in endothelial cells[23]. Pim-3 shRNA treatment impaired endothelial cell spreading, migration, and proliferation, leading to a reduction in tube-like structure formation in a Matrigel assay[23]. Moreover, tumor necrosis factor (TNF)-α transiently increases Pim-3 mRNA expression *via* the TNF receptor-1 pathway in endothelial cells (ECs), and eventually promotes EC spreading and migration[77]. Constitutive Pim-3 overexpression in gastric cancer tissues can induce angiogenesis[30].

Tumor-associated neovasculature formation is regulated by various angiogenic factors. Notably, vascular endothelial growth factor (VEGF) has an important role in tumor-associated vasculature formation[78,79]. Although most pancreatic cancer tissues are hypovascular, elevated levels of VEGF are sometimes detected in pancreatic cancer cells[80]. Earlier studies have demonstrated that Pim-3 overexpression was responsible for increased VEGF expression and the growth of pancreatic cancer *in vivo* in an orthotopic nude mouse model[81]. The lack of any vascular phenotypes in Pim-3-deficient mice indicates that Pim-3 is dispensable for normal vasculature formation. However, given distinctive gene expression profiles of tumor-associated ECs[82], Pim-3 may have distinct roles in tumor-associated endothelial cells.

PHARMACOLOGICAL CHARACTERIZATION OF PIM-3 INHIBITORS

It is obvious from our discussions that aberrant activation and expression of Pim kinases are associated with various types of cancer. Enhanced expression of Pim-2 kinase is detected in hematologic malignancies and prostate cancer. Additionally, increased Pim-1 expression is observed in pancreatic cancer, squamous cell carcinoma, gastric cancer, colorectal cancer, hepatocellular carcinoma[83-85], bladder carcinoma[86], and liposarcoma[87]. In contrast, Pim-3 expression is selectively overexpressed in malignant lesions of endoderm-derived organs such as the liver[13], pancreas[5], colon[29], and stomach[30]. Fur-

thermore, a lack of apparent phenotypes in TKO mice suggests that Pim kinases are dispensable for the maintenance of normal functions of vital organs. Collectively, Pim kinases can be good candidate molecules for targeted cancer therapy. Examples of Pim-1 inhibitors include an anti-Pim-1 antibody and a cell penetrating peptide, both of which suppresses tumor growth *in vivo* in xenograft mouse models transplanted with human cancer cell lines[88,89].

The crystal structure of Pim-3 has not yet been reported. However, the crystal structure of Pim-1 and Pim-2 has been resolved, which revealed the presence of a unique hinge region that connects the two lobes of the protein kinase domain[18-20]. As a result, ATP binds to Pim kinases in a fundamentally different way from how it binds to other protein kinases[18,19]. Thus it may be possible to design compounds which will selectively inhibit Pim kinases but not other serine/threonine kinases[16].

Several independent research groups have developed small-molecule inhibitors against Pim kinases, including flavonol quercetagetin[90], imidazole[1,2-b]pyridazines[91,92], benzylidene-thiazolidine-2,4-dione[93-95], 3,5-disubstituted indole derivatives[96], pyrazolo[3,4-g]quinoxaline derivatives[97], 1,6-dihydropyrazolo[4,3-c]carbazoles and 3,6-dihydropyrazolo[3,4-c]carbazole derivatives[98], and pyrrolo[2,3-a]carbazole and pyrrolo[2,3-g]indazole derivatives[99-101]. Among them, 1,6-dihydropyrazolo[4,3-c]carbazoles, 3,6-dihydropyrazolo[3,4-c]carbazoles, and pyrrolo[2,3-g]indazoles can inhibit Pim-3 activities[98,100]. In our previous studies, we have demonstrated that derivatives of stemoamide synthetic intermediates can inhibit Pim-3 as well as Pim-1 and Pim-2 activities, and can reduce tumor growth *in vivo* in mouse xenograft models using human pancreatic cancer cell line without causing major adverse side-effects[102,103].

The substrates preferred by Pim-1 and Pim-3[19] are very similar in identity. Therefore, designing isoform specific inhibitors that will differentiate and preferentially bind to one Pim member over the other is extremely challenging. Indeed, pyrrolo[2,3-*a*]carbazole has low nanomolar binding affinity for Pim-1 and Pim-3 kinases, but only weakly inhibits Pim-2 (IC_{50} for Pim-1, 0.57 + 0.04 μmol/L; IC_{50} for Pim-2, > 10 μmol/L; IC_{50} for Pim-3, 0.04 + 0.01 μmol/L)[104]. Similar pharmacological observations have been recorded with phenanthrene derivatives[77]. However, it will be interesting to find out if an inhibitor which specifically inhibits the action of one Pim member will provide any additional advantage over a multi-Pim kinase inhibitor.

Akt can phosphorylate a similar set of substrates to Pim kinases, such as BAD, thereby initiating the proliferation of cancer cells[105]. Akt is aberrantly activated in various types of tumors, and Akt inhibitors have been extensively investigated[72]. The Akt inhibitor "GSK690693" has exhibited potent antitumor activity in pre-clinical trials on animals[105]. Akt is a key signaling protein and Akt-2 is directly involved in the insulin receptor signal-

ing pathway. Consequently, the genetic disruption of Akt kinase genes results in severe phenotypic changes, such as neonatal mortality, severe growth retardation, and reduced brain size[106-108], and Akt-2 inhibition induces severe hyperglycemia[105]. The use of Akt inhibitors for anti-cancer treatment is seriously limited because of these shortcomings. In contrast, Pim kinases, including Pim-3, are not involved in the insulin receptor signaling pathway, and the inhibition of Pim kinases hardly shows any detrimental effects on normal glucose metabolism. Thus, Pim kinases are more effective molecular targets than Akt for targeted cancer therapy, and are particularly useful for treating pancreatic cancer, which is frequently complicated by hyperglycemia.

CONCLUSION

Pim-3 kinase is aberrantly expressed in malignant lesions but not in normal tissues of endoderm-derived organs, such as the liver, pancreas, colon, and stomach[5,13,29,30], and contributes to tumorigenesis by inhibiting apoptosis of tumor cells and promoting cell cycle progression. Moreover, genetic deficiency of the *Pim-3* gene does not result in apparent changes in phenotypes, suggesting that Pim-3 may be physiologically dispensable. Unlike Akt kinases[72], Pim kinases are not involved in the insulin receptor signaling pathway; therefore, the inhibition of Pim kinases has very little influence on glucose metabolism. Indeed, inhibition of Pim-3 kinase activities slows the growth or even causes regression of pancreatic tumors in mice without causing hypoglycemia[66,102,103]. Since Pim-3 kinase is constitutively active once it is expressed aberrantly, inhibition of Pim-3 can be used for inhibiting cancer progression. Furthermore, there is accumulating evidence to suggest that Pim-3 plays a vital role in the interaction between tumor cells and their surrounding stroma. Further studies on these aspects will unravel the novel pathophysiological role of Pim-3. Nevertheless, strategies to inhibit Pim-3 activity warrant intensive investigation in order to discover and develop new targeted anti-cancer therapeutics.

ACKNOWLEDGMENTS

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2014 ADVANCES IN PANCREATIC CANCER

Role of non-coding RNAs in pancreatic cancer: The bane of the microworld

Yi-Ting Tang, Xiao-Hui Xu, Xiao-Dong Yang, Jun Hao, Han Cao, Wei Zhu, Shu-Yu Zhang, Jian-Ping Cao

Yi-Ting Tang, Han Cao, Wei Zhu, Shu-Yu Zhang, Jian-Ping Cao, School of Radiation Medicine and Protection, Jiangsu Provincial Key Laboratory of Radiation Medicine and Protection and Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou 215123, Jiangsu Province, China

Xiao-Hui Xu, Xiao-Dong Yang, Department of General Surgery, The Second Affiliated Hospital of Soochow University, Suzhou 215004, Jiangsu Province, China

Jun Hao, Department of Pancreatic Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, China

Author contributions: Tang YT and Xu XH contributed equally to this work; Zhang SY, Cao JP and Zhu W designed the article; Tang YT, Xu XH and Yang XD wrote the paper; Hao J, Zhang SY and Cao H amended the paper.

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Correspondence to: Jian-Ping Cao, Professor, School of Radiation Medicine and Protection, Jiangsu Provincial Key Laboratory of Radiation Medicine and Protection, Medical College of Soochow University, 199 Ren'ai Rd, Suzhou 215123, Jiangsu Province, China. jpcao@suda.edu.cn

Telephone: +86-512-65880065 Fax: +86-512-65880065

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Abstract

Our understanding of the mechanisms underlying the development of pancreatic cancer has been greatly advanced. However, the molecular events involved in the initiation and development of pancreatic cancer remain inscrutable. None of the present medical technologies have been proven to be effective in significantly improving early detection or reducing the mortality/morbidity of this disease. Thus, a better understanding of the molecular basis of pancreatic cancer is required for the identification of more effective diagnostic markers and therapeutic targets. Non-coding RNAs (ncRNAs), generally including microRNAs and long non-coding RNAs, have recently been found to be deregulated in many human cancers, which provides new opportunities for identifying both functional drivers and specific biomarkers of pancreatic cancer. In this article, we review the existing literature in the field documenting the significance of aberrantly expressed and functional ncRNAs in human pancreatic cancer, and discuss how oncogenic ncRNAs may be involved in the genetic and epigenetic networks regulating functional pathways that are deregulated in this malignancy, particularly of the ncRNAs' role in drug resistance and epithelial-mesenchymal transition biological phenotype, with the aim of analyzing the feasibility of clinical application of ncRNAs in the diagnosis and treatment of pancreatic cancer.

Core tip: The deregulation mechanisms of pancreatic cancer remain inscrutable. ncRNAs have recently been found to provide new opportunities for identifying both functional drivers and specific biomarkers of pancreatic cancer. Here, we review the expression profile of ncRNAs in human pancreatic cancer, with the aim of analyzing the feasibility of clinical application of ncRNA in pancreatic cancer's diagnosis and treatment.

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INTRODUCTION

Pancreatic cancer is a lethal malignancy with poor prognosis due to advanced stage disease at initial diagnosis, frequent recurrence and the absence of treatment strategies that specifically and effectively target these tumors[1]. Only 15% of pancreatic adenocarcinoma patients are candidates for surgical resection at the time of diagnosis[2]. Chemotherapy is considered the main treatment option for unresectable cases, while chemo-radiotherapy may improve survival and quality of life[3,4]. However, even with advancements in medicine, pancreatic cancer is still extremely resistant to the currently available regimens. The burden of pancreatic disorders is expected to increase over time[5]. This situation represents a challenge for doctors as well as scientists in seeking the best active regimen with the least side effects[6]. Therefore, there is an urgent need to understand the molecular mechanisms underlying this disease, including the genetic and epigenetic networks influencing the malignant transformation, metastasis and chemo-resistance mechanisms of pancreatic cancer.

In recent years, it has become increasingly apparent that the non-protein-coding portion of the genome is of crucial functional importance: in relation to both normal physiology and diseases[7]. The relevance of the non-coding genome to human disease has mainly been studied in the context of the widespread disruption of miRNA expression and function in human cancers. Research is also being conducted aimed at understanding the nature and extent of other ncRNAs in disease, such as PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), transcribed ultraconserved regions (T-UCRs) and large intergenic non-coding RNAs (lincRNAs)[7]. Along with miRNAs, dysregulation of these ncRNAs is being found to show key relevance to pancreatic tumorigenesis. Consequently, exploring ncRNAs as therapeutic targets and biomarkers for the diagnosis and prognosis of pancreatic cancer is of interest.

MECHANISMS OF PANCREATIC CANCER DEREGULATION

The pancreas is a glandular organ of both the digestive system and endocrine system of vertebrates and displays an astonishing capacity to carry out cellular functions. Pancreatic dysfunction can be highly deleterious. As the fourth leading cause of cancer-related death in United States and worldwide, pancreatic cancer continues to remain a devastating disease with only 5.2% of patients alive for more than 5 years[8]. Genetic analysis of pancreatic cancer indicated that multiple mutations accumulate over time with some of them being more frequent than others [such as KRAS (about 90%), p16/CDKN2A (about 75%), TP53 (about 65%), SMAD4 (about 50%)] in these tumors[9,10]. However, blocking the activity of these frequently mutated genes did not turn out to be a promising therapeutic strategy[11]. Identification of mo-

lecular mechanisms that are more directly associated with the sustained proliferation and aggressive of pancreatic cancer cells is urgently needed. In recent years it has been proposed that in addition to the accumulated mutations that favor cancerous growth, epigenetic events may also play an important role in the development and maintenance of pancreatic cancer[12]. ncRNAs are already known as master epigenetic regulators that have a role in regulating diverse cellular processes including cell proliferation, development, differentiation, apoptosis and consequently oncogenesis. In view of the differentially expressed oncogenic and tumour suppressor ncRNAs targeting multiple genes in important cancer-relevant genetic and epigenetic networks in pancreatic cancer, it is logical to suggest that distinct ncRNA expression signatures may be associated with different grades and stages and meanwhile provide therapy strategy for this malignancy.

TYPES OF NCRNAS AND THEIR FUNCTIONS

RNA is the bridge between stable DNA and versatile proteins. Traditional studies are mainly focused on protein-coding RNAs, however, the coding exons of genes account for only 1.5% of the genome[13]. With the development of new scientific technologies, especially concerning the widespread use of microarray technology in molecular biology research, significant portions of eukaryotic genomes have been found to give rise to non-protein-coding RNAs, many of which remain unannotated.

In general, ncRNAs are grouped into two major classes based on their length (Table 1). Transcripts shorter than 200 nucleotides (nt) are usually referred to as small ncRNAs, which include miRNAs, Piwi-interacting RNAs, small-interfering RNAs and some bacterial regulatory RNAs. The well-documented about 22 nt long miRNAs serve as important regulators of gene expression and as intricate components of the cellular gene expression network[14-16]. lncRNAs are mRNA-like transcripts ranging in length from 200 nt to 100 kb; they are poorly conserved and do not function as templates for protein synthesis. ncRNAs have recently been discovered to act as robust regulators of gene expression that are frequently deregulated in human cancers, providing new opportunities to unravel the aberrantly expressed cellular pathways. However, as noted above, while the biological functions and molecular mechanisms of ncRNAs have been widely investigated in cancer studies (Figure 1), their prognostic value associated with pancreatic cancer remains to be elucidated.

NCRNAS IN PANCREATIC CANCER

Although all types of ncRNAs are transcribed in human cells, most of the current findings concerning ncRNAs are focused on miRNAs. Additionally, lncRNAs are also gaining prominence as emerging key elements of cel-

Table 1 Types of non-coding RNAs in pancreatic cancer and their functions

Types	Name	Expression profile	Related biological function
Short ncRNAs			
miRNAs	Let-7[117]	Up	Downregulates STAT3 phosphorylation
	miR-15/16[17,18]	Up	Promoting tumor angiogenesis
	miR-34a[29]	Down	Inactivates p53 and modulates pancreatic cancer pathogenesis
	miR-132[88]	Down	Transcribed by RNA polymerase Ⅱ and promoter methylation
	miR-200[36]	Down	Leading to drug resistance
	miR-421[30,31]	Up	Suppresses expression of DPC4/Smad
piRNAs	pi-651[42]	Up?	piRNAs target transposon repression and DNA methylation
snoRNAs	U50, SNORD[46,118]	Up?	
Long ncRNAs			
lincRNAs	PPP3CB/MAP3K1 4/DAPK1 loci	Up	Metastases associated with the MAPK pathway[69]
	HOTAIR[73]	Up	Associates with the polycomb repressive complex 2
Other lncRNAs	HSATII (satellite repeat RNAs)[70]	Up	Reflect global alterations in heterochromatin silencing

HOTAIR: Homeobox transcript antisense RNA; MAPK: Mitogen-activated protein kinase; STAT3: Signal transducer and activator of transcription 3.
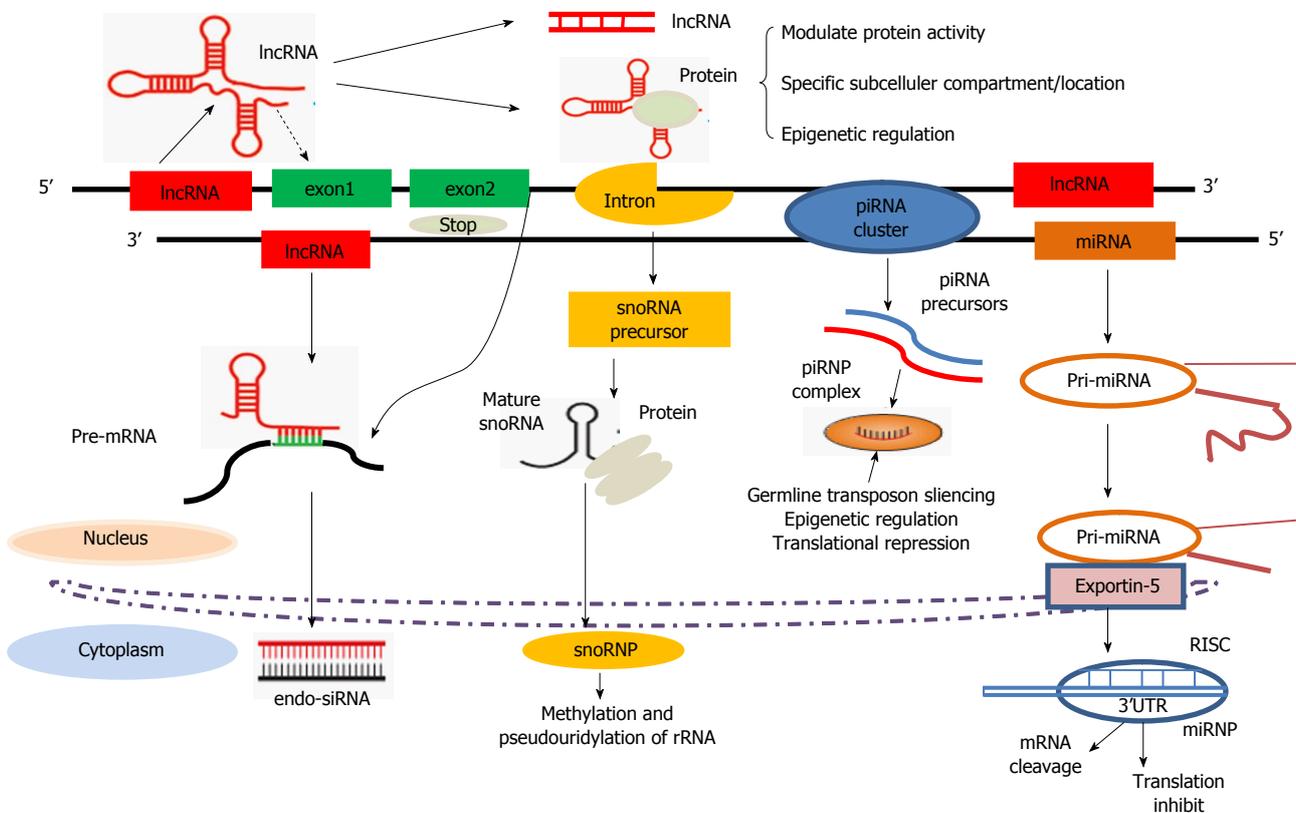
Figure 1 Biogenesis and functional machinery of small ncRNAs (miRNAs, piRNAs, snoRNAs) and lncRNAs. (1) miRNAs: pri-miRNA is transcribed by RNA polymerase Ⅱ, then cleaved by Droshain into pre-miRNA. Pre-miRNAs are then transported to the cytoplasm *via* an Exportin-5-mediated mechanism and further processed by the RNase Ⅲ endonuclease into mature miRNAs. Mature miRNAs are then incorporated into the RISC, where they direct the complex to specific mRNAs through complementary base pairing in their 3'UTRs; (2) piRNAs: piRNAs are mainly expressed from piRNA clusters. Subsequently, additional piRNAs influence the functions of ribonucleoprotein (piRNP) complexes in transposon repression through target degradation and epigenetic silencing; (3) snoRNAs: snoRNAs are transcribed from introns. Following splicing, debranching and trimming, mature snoRNAs are either exported, in which case they function in rRNA processing, or remain in the nucleus, where they are involved in alternative splicing; and (4) lncRNAs: lncRNAs exhibit a 5' terminal methylguanosine cap and are often spliced and polyadenylated. lncRNAs contain structural domains that can sense or bind to other RNAs *via* complementary base pair interactions, in addition to interacting with proteins and possibly DNA and displaying other as yet unknown functions. endo-siRNA: Endogenous small-interfering RNA; lncRNA: Long non-coding RNA; miRNA, microRNA; piRNA: PIWI-interacting RNA; pre-miRNA: Primary miRNA; pre-miRNA: Precursor miRNA; rRNA: Ribosomal RNA; RISC: RNA-induced silencing complex; sncRNA: Small non-coding RNA; snoRNA: Small nucleolar RNA; 3'UTRs: 3' untranslated regions.

lular homeostasis (Table 1), which draws our attention toward emphasizing the role of miRNAs and lncRNAs in pancreatic cancer development and progression in this review.

miRNAs in pancreatic cancer

miRNAs The most widely studied class of ncRNAs are miRNAs, which are small ncRNAs of 22 (18-25) nt in animals that mediate post-transcriptional gene silenc-

ing by controlling the translation of mRNAs into proteins[17,18]. Following the discovery that in *C. elegans*, the small RNAs encoded by the lin-4 gene are associated with the control of developmental timing through negatively regulating lin-14 translation[19], there was an explosion in the field of small ncRNA biology in subsequent years across different species. The biogenesis of miRNAs involves several steps (Figure 1). First, a several kilobase-long primary RNA (pri-miRNA) is transcribed by RNA polymerase Ⅱ. The pri-miR is then cleaved by Droshain (an RNase Ⅲ endonuclease) into a hairpin loop structure known as the precursor miRNA (pre-miRNA), after which the pre-miR is transported to the cytoplasm *via* a RanGTP-dependent Exportin-5-mediated mechanism, where it is further processed by the RNase Ⅲ endonuclease into a 17-25 nt-long mature duplex miRNA. Mature single-stranded miRNAs are then incorporated into the RNA-induced silencing complex (RISC) and direct the complex to specific mRNAs through complementary base pairing with their 3' untranslated regions (3'UTRs). Partial complementarity between the 3'UTR of the target gene and the seed region of the miRNA leads to mRNA decay and/or inhibition of translation[17,20]. It is predicted that on an average, each miRNA may regulate about 200 gene transcripts[21].

miRNAs in pancreatic cancer development and progression: miRNAs have emerged as critical components of networks of complex functional pathways controlling important cellular processes, such as proliferation, differentiation, apoptosis, stress response and drug resistance[22]. Recent analyses of tumor miRNA expression profiles have demonstrated that the miRNAs that are abnormally expressed in tumors usually exhibit reduced expression levels compared to their expression levels in normal tissues[23]. The role of miRNAs in tumorigenesis and tumor development has recently been reviewed in detail elsewhere[24], and it is therefore only briefly recapped here. First, downregulated expression of a tumor suppressor miRNA can lead to the expression of miRNA target genes that promote tumorigenesis, causing excessive cell proliferation and abnormal differentiation and resulting in tumorigenesis. On the other hand, the overexpression of oncogenic miRNAs can lead to decreased expression of target genes with tumor suppressor functions, thereby promoting tumor initiation and development. Another role of oncogenic miRNAs is the regulation of tumor angiogenesis. The cellular levels of miR-15b and miR-16 are downregulated under hypoxic conditions, leading to diminished inhibitory effects of miR-15b and miR-16 on VEGF and thereby promoting tumor angiogenesis[25]. miRNAs such as miR-10b and miR-373 are also important drivers of tumor metastasis[26,27]. A number of expression profiling studies have demonstrated that deregulation of miRNA expression occurs in pancreatic cancer tissues and cell lines. An extensive analysis of miRNA expression profiles in tissue samples from normal pancreases and patients

with chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC) using microarray technology revealed that some miRNAs, including miR-29c and miR-96, were differentially expressed in both the chronic pancreatitis and PDAC samples, whereas the expression of miR-196s, miR-203 and miR-210 was altered only in PDAC tissues[28]. Moreover, p53 inactivation contributes to the reduction of miR-34a levels, which raises the possibility that loss of miR-34a function modulates the pathogenesis of pancreatic cancer[29]. Our previous findings have identified miR-421 and miR-483-3p as potent regulators of DPC4/Smad4, which may provide a novel therapeutic strategy for the treatment of DPC4/Smad4-driven pancreatic cancer[30,31]. Taken together, these findings indicate that miRNAs play an important role in the development and progression of pancreatic cancer.

miRNAs in pancreatic drug resistance: Chemotherapy represents an important therapeutic strategy for most patients with pancreatic cancer. Prior to the 1990s, 5-fluorouracil (5-FU) was the accepted monotherapy. However, soon after Burris and colleagues reported the results of a phase Ⅲ clinical trial directly comparing gemcitabine and 5-FU[3], showing that gemcitabine confers significantly increased median survival, gemcitabine became the first-line treatment and gold standard for pancreatic cancer chemotherapy. Nevertheless, drug resistance can cause failure of this treatment[6]. Despite investigations into the mechanisms underlying drug resistance over the past 50 years, much is still unknown about exactly how this phenomenon occurs. The three most common reasons for the acquisition of drug resistance are the expression of energy-requiring transporters, insensitivity to drug-induced apoptosis and the induction of drug-detoxification mechanisms[32]. The tumor cell microenvironment (*e.g.*, interactions between cell surface integrins and extracellular matrix components) is responsible for innate drug resistance[33]. miRNAs appear to be critical regulators of drug resistance in pancreatic cancer cells[34]. For example, in one study, the levels of the oncogenic miR-155 were shown to increase after pancreatic cancer cells were treated with gemcitabine[35]. miR-200a, miR-200b and miR-200c are all downregulated in pancreatic cancer cells that are resistant to gemcitabine[36]. A recent report suggests that miR-34 is involved in the self-renewal of pancreatic cancer stem cells, while the loss of miR-34 in pancreatic cancer is associated with an enrichment of cancer stem cells that are insensitive to chemotherapy[37] (Figure 2). Evidence also indicates that miRNAs might regulate the epithelial-mesenchymal transition (EMT) through the regulation of cadherin1 and other molecules[38], which mediate various types of cellular drug-resistance mechanisms (Figure 2). Many members of the Let-7 family are downregulated in EMT-type cells that are resistant to gemcitabine. In an investigation of the expression levels of miR-200 and Let-7 in EMT-phenotype pancreatic cancer cells that are resistant to gemcitabine, re-expression of the downregulated miR-200 family upregulates
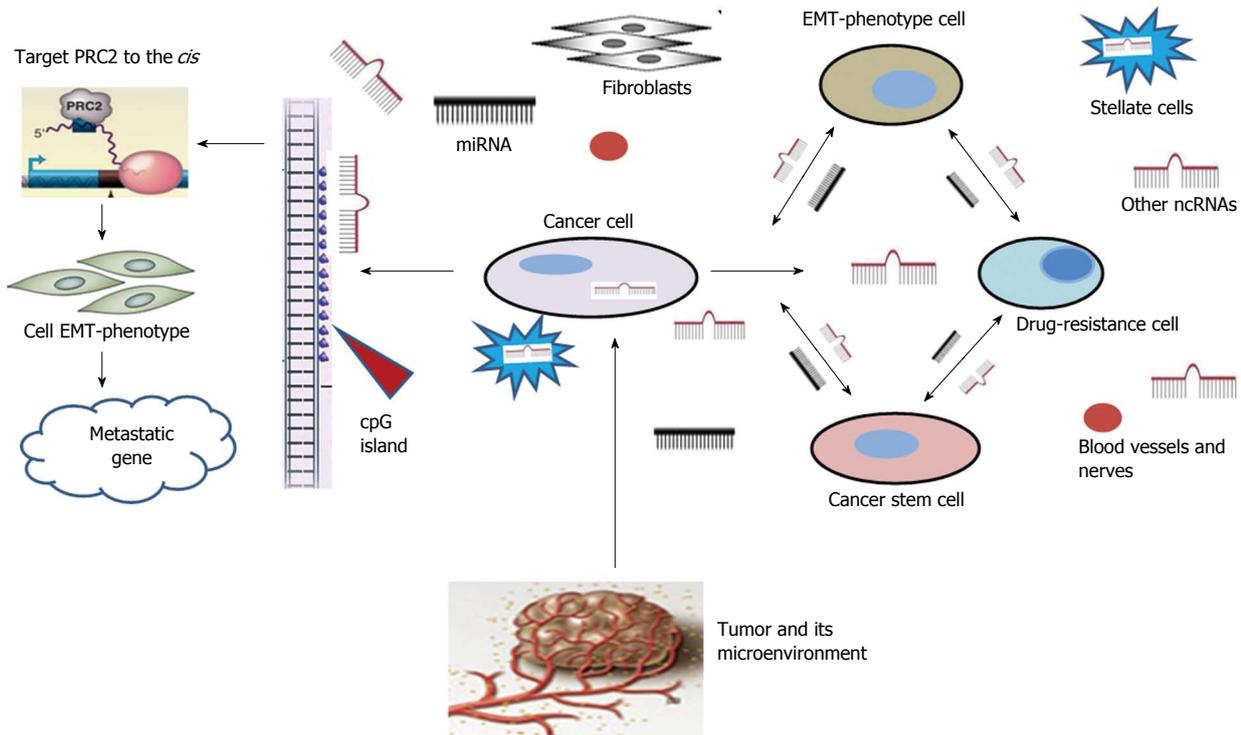
Figure 2 Pancreatic cancer cell microenvironment (the cellular elements are predominately mesenchymal cells such as pancreatic stellate cells, fibroblasts, blood vessels and nerves) and the links between cancer cells and drug-resistant cells determined through the epithelial-mesenchymal transition-phenotype as well as between cancer stem cells and the ncRNAs in their surroundings. PRC2: Polycomb repressive complex 2; EMT: Epithelial-mesenchymal transition.

cadherin1 and downregulates ZeB1 and vimentin (EMT inducers). Knockdown of ZeB1 in mesenchymal Panc1 cells results in the upregulation of miR-200c[36]. Therefore, re-expression of specific miRNAs could serve as a new strategy for the treatment of pancreatic cancer *via* the targeted elimination of either cancer stem cells or EMT-phenotype cells that contribute to tumor recurrence and metastasis.

Other small ncRNAs in pancreatic cancer

Other ncRNAs, such as piRNAs and snoRNAs, are also reported to be associated with cancer. Thousands of different piRNA species have been found in mammals[39]. piRNAs are generated from single-stranded RNA precursors through a Dicer-independent mechanism[40], providing essential protection for germ-cell genomes against the activity of transposable elements[41] (Figure 1). Regarding the role of piRNAs in cancer, they were first described to be over-expressed in seminomas but not in non-seminomas of the adult testis. Recent studies have highlighted the role of piRNAs in the regulation of tumorigenesis. For example, the expression of piR-651 in gastric, colon, lung, and breast cancer tissues is higher compared to normal adjacent tissues[42]. The levels of piR-651 have also been associated with the stage of tumor-node-metastasis (TNM)[43].

snoRNAs are small non-coding RNAs with lengths ranging from 60 to 300 nt. snoRNAs are normally located within the introns of protein-coding genes and are

transcribed by RNA polymerase Ⅱ (Figure 1), although in some cases, they can be found within the introns of lncRNAs. snoRNAs and snoRNPs are likely to contribute to tumorigenesis through an effect on ribosomes and protein translation, and snoRNAs might also be involved in the regulation of gene expression by giving rise to other regulatory RNA species, such as miRNAs[44]. Initial insights into the potential roles of snoRNAs in cancer were provided by a study in which substantial downregulation of snoRNAs was observed in meningiomas compared with normal brains[45]. Other studies have shown that a germline homozygous 2 bp (TT) deletion in the snoRNA U50 is associated with the development of prostate cancer[46]. However, the mechanisms through which both piRNAs and snoRNAs function to target pancreatic cancer remain unknown. The currently available data suggest important roles of piRNAs and snoRNAs that go beyond the regulation of the genome in germline tissues, and further studies are needed to reveal their specific roles in pancreatic tumorigenesis.

lncRNAs in pancreatic cancer

An updated view of lncRNAs: lncRNAs are a heterogeneous group of non-coding transcripts more than 200 nt long. This class of ncRNAs makes up the largest portion of the mammalian non-coding transcriptome[7]. Although the proverbial "dark matter" of the genome was initially argued to be spurious transcriptional noise, recent evidence suggests that it may play a major bio-

logical role in cellular development, differentiation and metabolism[47]. lncRNAs often overlap with, or are interspersed between, multiple coding and non-coding transcripts[48,49]. Many transcripts resist classification into any particular category and instead exhibit a combination of these qualities. Based on their genomic proximity to protein-coding genes, lncRNAs are generally classified into five types: (1) sense; (2) antisense; (3) bidirectional; (4) intronic; and (5) intergenic[7,50]. In many cases, the secondary structure of lncRNAs dictates their function. For example, conservation of the secondary structure of the lncRNA maternally expressed gene 3 (MEG3) and its propensity to fold into thermodynamically stable secondary and higher-order structures maintains the tumor suppressor function of this lncRNA, rather than its primary sequence[51]. By virtue of their ability to base pair with other RNAs, lncRNAs can act as highly specific sensors of the expression of mRNAs, miRNAs and other lncRNAs (Figure 1). The conversion of modified nucleotides for detection *via* high throughput sequencing is currently revealing widespread nucleotide modifications throughout the transcriptome[52]. There is a growing catalog of bifunctional mRNAs: coding transcripts can lose their ability to encode a protein, and noncoding transcripts can acquire a coding function[53-55]. Many of these posttranscriptional modifications are reversible, and given the range of modifications and targets observed, they may constitute an additional layer of posttranscriptional regulation, analogous to the epigenetic landscape regulated by lncRNAs[56].

There are three types of well-known lncRNAs: homeobox transcript antisense RNA (HOTAIR), lincRNAs and T-UCRs. lncRNAs are essential in many physiological processes, such as X-chromosome inactivation in mammals, in which the X-inactivation-specific transcript (XIST) lncRNA (17 kb) recruits the polycomb complex to silence the X chromosome from which it is transcribed[57]. lincRNAs, which are transcribed from intergenic regions, are associated with active transcription in the regions across which transcriptional elongation takes place[58]. Thus far, the aberrant regulation of T-UCR expression in cancers has been found to occur in two main ways: through altered interactions with miRNAs[59] and *via* hypermethylation of CpG islands in their promoters[60]. Among various examples of the involvement of lncRNAs in cancer, the role of HOTAIR in human neoplasia is the best understood[61]. HOTAIR might play an active role in modulating the cancer epigenome and mediating cell transformation[61].

lncRNAs and pancreatic cancer: Although the functional annotation of lncRNAs on a genomic scale has remained elusive for quite some time, lncRNAs may play critical roles in the regulation of multiple biological processes[62], including epigenetic regulation, transcriptional regulation, the processing of small RNAs, and other regulatory functions[63-65]. Due to advancements in cancer transcriptome profiling and the accumulating evidence

indicating the functions of lncRNAs, these ncRNAs are emerging as new factors in the cancer paradigm, showing potential roles in both oncogenic and tumor-suppressive pathways[66]. Recent studies have demonstrated that certain lncRNAs are specifically associated with certain types of cancer, and their expression level may function as an indicator of metastasis or prognosis[61,67,68]. Despite the many lncRNAs that have been identified or validated in human cancer tissues, there is a paucity of information regarding the expression of lncRNAs in pancreatic cancer. Tahira *et al*[69] were the first to perform a microarray interrogation of protein-coding genes and putative lncRNAs and indicated that subsets of intronic/intergenic lncRNAs are expressed in both pancreatic tumor and non-tumor tissue samples. They identified loci harboring intronic lncRNAs (PPP3CB, MAP3K1 4 and DAP K1 loci) that were differentially expressed in PDAC metastases and were enriched in genes associated with the MAPK pathway. In an interesting study performed by Ting *et al*[70], aberrant overexpression of satellite repeat RNAs (HSAT Ⅱ) ranging from 100 to 5000 nt was observed in patients with PDAC. The overexpression of satellite transcripts in cancer may reflect global alterations in heterochromatin silencing and could potentially be useful biomarkers for pancreatic cancer detection[70].

HOTAIR is a long intervening non-coding RNA (lincRNA) that associates with the polycomb repressive complex 2 (PRC2), and its overexpression is correlated with poor survival in breast, nasopharyngeal and liver cancer patients[61,71,72]. Kim *et al*[73] showed that knockdown of HOTAIR in PANC1 and L3.6pL pancreatic cancer cells decreases cell proliferation, alters cell cycle progression and induces apoptosis, demonstrating an expanded function of HOTAIR in pancreatic cancer cells. HOTAIR is a negative prognostic factor for pancreatic cancer patients and exhibits pro-oncogenic activity in both *in vitro* and *in vivo* bioassays.

Nevertheless, only a small number of lncRNAs have been well characterized in pancreatic cancer, and little of the underlying molecular mechanism has been elucidated. The potential importance of lncRNAs in pancreatic cancer demands further study.

ncRNAs are linked to epigenetic mechanism of pancreatic cancer in the microenvironment

Pancreatic cancer and its microworld: Several lines of evidence indicate that tumorigenesis is a complex and multistep process. Cancers acquire the same set of functional capabilities during development and progression through various mechanistic strategies. The importance of the local tumor microenvironment to tumor progression has been recognized for many years and was highlighted in several reviews[74-76]. The cellular elements of pancreatic tumor environment are predominately mesenchymal cells such as pancreatic stellate cells, fibroblasts, blood vessels and nerves (Figure 2). Using *in vitro* and *in vivo* models containing tumor cells and stromal cells, investigators have shown that stromal signals and cell-

to-cell interactions including proteases, growth factors, and mediators of invasion are critical determinants of pancreatic cancer behavior[77,78]. As pancreatic cancer has an especially abundant stroma originating from abundant pancreatic stellate cells, lacking of suitable models limited the exploration of interactions between pancreatic cancer cells and the adjacent stromal cells[79]. It was found that conditioned medium from human pancreatic stellate cells (HPSCs) stimulated pancreatic tumor cell proliferation and metastasis *in vitro* and *in vivo via* mitogen-activated protein kinase (MAPK) and Akt pathways[80]. However, simple models composed of cancer cells and their adjacent stromal cells in the tumor progress have several important limitations. Fortunately, with the development of ncRNA detection techniques, measurable biologically ncRNAs secreted from variational stroma complex provided novel research models in the exploration of role of tumor microenvironment in pancreatic carcinogenesis.

Epigenetics and pancreatic cancer: Epigenetics is a term used to describe mitotically and meiotically heritable states of gene expression that are not due to changes in DNA sequences[81], such as DNA methylation and histone tail modifications. Epigenetic regulations are important in all aspects of biology[82], and studies conducted during the past decade have shown that they play a key role in carcinogenesis and tumor progression[83]. It is evident that multiple epigenetic mechanisms are crucial in the development and progression of PDAC. In addition to genetic changes, epigenetic alterations add another layer of complexity and contribute to the heterogeneity of PDAC. Ongoing studies on chromatin dynamics are revealing the existence of robust machinery that can mediate epigenetic changes in pancreatic cells[12]. Epigenetic biomarkers, such as miRNAs, DNA methylation and satellite repeats, can be utilized to assess pancreatic cancer risk, progression and therapeutic responses; the use of these markers is regarded as the road to the early detection of pancreatic cancer. Numerous drugs that target specific enzymes involved in the epigenetic regulation of gene expression are emerging as an effective and valuable approach to chemotherapy[84]. A noteworthy characteristic of epigenetic-based inheritance is its reversibility, which contrasts with the stable nature of DNA sequence-based alterations.

ncRNAs are linked to epigenetic mechanisms associated with the biological phenotype of pancreatic cancer: Epigenetics influence genomic inheritance *via* miRNA-dependent mechanisms as well as DNA and histone modifications[12]. Members of the miR-200 family have been identified as modulators of the EMT due to negative activity against zinc finger E-box-binding homeobox1 and 2 (ZEB1 and ZEB2), which function as repressors of EMT-opposing genes[38,85,86]. The miR-34 family has been shown to be primarily inactivated by aberrant CpG methylation in PDAC, revealing an interesting example of an epigenetic mechanism influencing

the functioning of other mechanisms[87]. We have reported that the downregulation of miR-132 in pancreatic cancer tissues is due to promoter methylation, which consequently impairs the binding of essential transcription factors[88]. miR-107 and miR-148a have also been observed to be hypermethylated in the promoter region and, thus, down-regulated in pancreatic cancer[89,90]. Using a pyrosequencing technique, Wang *et al*[91] showed that the promoters of miR-124 family members (miR-124-1, miR-124-2 and miR-124-3) are highly methylated in pancreatic cancer tissues compared with non-cancerous tissues, and *in vitro* studies have shown that miR-124 inhibits cell proliferation, invasion and metastasis.

Important roles of lncRNAs have been described in epigenetic processes, and this broad topic has been reviewed elsewhere[92]. Lee noted that lncRNAs are implicated in almost every epigenetic regulation event[93]. The intriguing story of lncRNAs was initially related to the phenomena of genomic imprinting and X-chromosome inactivation[94,95]. The lncRNA AS1DHRS4 is transcribed from the locus of the *dehydrogenase/reductase SDR family member 4 (DHRS4)* gene and recruits DNA methyl transferases and other factors to the DHRS4 gene cluster, inducing DNA methylation in the DHRS4L2 promoter region[96]. Two other lncRNAs have been reported to induce either DNA methylation in specific regions of the Kcnq1 locus or demethylation at the Sphk1 CpG island[97,98]. Another potential mechanism is that lncRNAs recruit demethylases and/or acetylases to the promoter regions of oncogenes, and thus, the lncRNAs might direct the transcriptional activation of such protein-coding genes[99]. Some potential epigenetic mechanisms involving lncRNAs related to pancreatic cancer are as follows[99]: (1) lncRNAs affect DNA methylation; (2) lncRNAs alter nucleosome positioning; and (3) lncRNAs display an in-cis function or carry out trans-regulation.

It is evident that multiple epigenetic mechanisms are indeed crucial in the development and progression of pancreatic cancer. As one category of epigenetic alterations, our understanding of ncRNA-epigenetic-based events provides a new research model for studying PDAC.

NCRNA-BASED DIAGNOSIS AND TREATMENT OF PANCREATIC CANCER

Regarding the identification of molecular markers for pancreatic cancer diagnosis/prognosis, some promising candidate genes have been proposed[100]. However, none of these candidates have been proven to be effective in significantly improving early detection or reducing the mortality/morbidity of this disease. Thus, a better understanding of the molecular basis of pancreatic cancer is required for the identification of more effective diagnostic markers and therapeutic targets.

Not only are numerous miRNAs preferentially expressed between pancreatic cancer tissues and normal tissues, they are also extremely stable in both blood plasma

and serum[101]. Studies have demonstrated that miRNAs that are differentially expressed in PDAC can be profiled in blood as a minimally invasive biomarker assay for pancreatic cancer[102]. Consequently, miRNA levels serve as suitable tumor markers and biomarkers for testing conducted for clinical diagnosis. It has been suggested that endocrine tumors of the pancreas can be distinguished from acinar-type tumors based on a set of 10 miRNAs. These miRNAs are potentially associated with normal endocrine differentiation or endocrine tumorigenesis[103], indicating the diagnostic utility of miRNA expression signatures in pancreatic cancer. Another report addressing serum samples from pancreatic cancer patients and matched cancer-free controls indicates that numerous miRNAs display significantly different expression levels and present high sensitivity and specificity for distinguishing the various stages of pancreatic cancer compared to cancer-free controls[104]. miR-210 and miR-1290 were subsequently found to show ideal diagnostic performance in a qRT-PCR miRNA array analysis of sera from pancreatic cancer patients and controls[105,106]. A recent study analyzed the expression of several miRNAs in different types of pancreatic disease to determine if miRNA expression could aid in the diagnosis of PDAC and its precursor, pancreatic intraepithelial neoplasm (PanIN)[107]. Results showed that compared to the non-neoplastic parenchyma, miR-148a was significantly underexpressed, whereas miR-196a and miR-10b were highly overexpressed in PanIN. miR-217 expression was shown to decrease in PanIN compared to that in the non-neoplastic tissue[107], suggested that these miRNA markers may be involved in an early event in pancreatic carcinogenesis. These findings provide compelling reasons to pursue the targeting of miRNAs as novel molecular diagnostic markers and for use in therapeutic approaches in the early stage of pancreatic carcinogenesis.

The knowledge that miRNAs regulate their targets through base pairing has led to the use of antisense oligonucleotides (ASOs) to inhibit miRNA function therapeutically. ASOs inhibit miRNAs based on base pair complementarity. Three main classes of ASOs have been developed: locked nucleic acids (LNAs), anti-miRNA oligonucleotides (AMOs) and antagomirs, which incorporate different chemical modifications to increase their stability and efficacy[108-110]. However, as cancer progression is influenced by multiple miRNAs, recent research in this field suggests that several miRNAs can be simultaneously inhibited using mixed ASOs targeting multiple miRNAs[111]. One multiple-target anti-miRNA AMO was designed to suppress miR-21, miR-155 and miR-17-5p, which are oncogenic miRNAs that are frequently overexpressed in many types of tumors, showing promising results related to inhibiting cancer growth.

Dozens of lncRNAs have been found to be dysregulated in pancreatic cancer, raising the possibility that lncRNAs may represent promising biomarkers for the diagnosis and prognosis of this disease. The strategy of inhibiting the function of deregulated miRNAs could

also be useful for lncRNAs. The progress in the use of RNAi-mediated gene silencing for the treatment of different diseases is encouraging, as this strategy provides a straightforward approach for selectively silencing oncogenic lncRNAs. Thus far, few lncRNAs have been characterized as potential biomarkers in human body fluids. For example, the lncRNA PCA3 has been demonstrated as a more specific and sensitive marker of prostate cancer in urine samples from patients compared to the widely used serum prostate-specific antigen (PSA)[112,113], highlighting the advantages of PCA3 over PSA and enabling noninvasive diagnosis. Targeting human H19 for the treatment of bladder cancer with a plasmid-based system was found to be successful[114]. Decreased expression of the lncRNA HOTAIR has been shown to alter cell cycle progression and induce apoptosis, displaying a unique association with the treatment of pancreatic cancer patients, and overexpression of HOTAIR may serve as a potential biomarker of pancreatic cancer prognosis[73]. Circulating lncRNAs may be promising biomarkers for cancer diagnosis, and knockdown of lincRNAs can be achieved using siRNAs[61,115]. However, successful inhibition of long ncRNAs appears to be more difficult than inhibiting miRNAs, presumably due to the extensive secondary structure of lncRNAs. It is therefore urgent to ask important questions such as the following[66]: (1) How stable are circulating lncRNAs, and is their stability altered in various disease states? (2) although the RNA contents of microvesicles and exosomes reported thus primarily consist of small miRNAs, is it possible that long protein-coding mRNAs and lncRNAs are also packaged into microparticles in a manner similar to miRNAs? (3) as considerable numbers of circulating lncRNAs may be dysregulated in various human diseases, might these lncRNAs be causing the disease, or do they become altered as a consequence of the disease itself? These ncRNAs could represent an unexpected source of potential diagnostic and prognostic biomarkers that might aid in addressing the great challenges related to the battle against pancreatic cancer[66].

However, the overall treatment strategy is to correct imbalances of ncRNAs (Figure 3), including drugs or adenovirus-associated vectors enhancing ncRNA processing or synthetic-anti ncRNAs, epigenetic ncRNA silencing or other strategies. ncRNAs may represent a gold mine for all diagnostic and therapeutic strategies. More studies need to be conducted to confirm this phenomenon and how these ncRNAs are involved in the early stage of pancreatic carcinogenesis.

CONCLUSION

The outlook for patients with PDAC is bleak, primarily because these tumors are detected late and are often too advanced for surgical resection[116]. The number of patients who die annually from pancreatic cancer continues to increase, despite an overall decrease in cancer deaths worldwide. It is our goal to be able to reduce the local
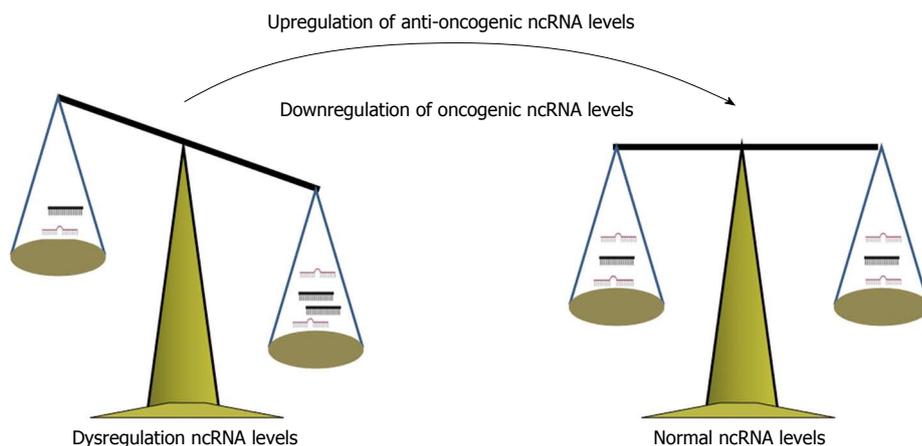
Figure 3 Overall therapeutic strategy is to correct imbalances of ncRNAs, including inhibiting/restoring their functions *via* biological and/or biochemical techniques. ncRNA: Non-coding RNA.

invasiveness of pancreatic cancer, allow more patients to undergo curative surgical resection and prevent local recurrence, which is observed in 80% of pancreatic cancer patients. Research on ncRNAs has not only increased greatly but has changed in character during the past quarter century, providing new research methodology to improve our understanding of the biological functions, molecular mechanisms and prognostic value associated with pancreatic cancer in the curable stage.

A major challenge remains regarding the elucidation of the biological pathways or signaling networks underlying cancer development and how specific ncRNAs interact or contribute to malignant transformation. There is increasing evidence suggesting an association of ncRNAs with disease, and knowledge of disease-related ncRNAs is essential in relation to the pathogenesis of pancreatic cancer at the molecular level. We must also continue to expand our research into the associated genetic and epigenetic mechanisms to obtain complete picture regarding the alterations of gene expression that occur in pancreatic cancer.

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2014 ADVANCES IN PANCREATIC CANCER

Rapid on-site evaluation of endoscopic-ultrasound-guided fine-needle aspiration diagnosis of pancreatic masses

Julio Iglesias-Garcia, Jose Lariño-Noia, Ihab Abdulkader, J Enrique Domínguez-Muñoz

Julio Iglesias-Garcia, Jose Lariño-Noia, J Enrique Domínguez-Muñoz, Department of Gastroenterology and Hepatology, Foundation for Research in Digestive Diseases, University Hospital of Santiago de Compostela, 15706 Santiago de Compostela, Spain

Ihab Abdulkader, Department of Pathology, University Hospital of Santiago de Compostela, 15706 Santiago de Compostela, Spain

Author contributions: Iglesias-Garcia J made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content; Lariño-Noia J and Abdulkader I drafted the article and revised it critically for important intellectual content; Domínguez-Muñoz JE gave final approval of the version to be published.

Correspondence to: Julio Iglesias-Garcia, MD, PhD, Department of Gastroenterology and Hepatology, Foundation for Research in Digestive Diseases, University Hospital of Santiago de Compostela, 15706 Santiago de Compostela, Spain. julio.iglesias.garcia@sergas.es

Telephone: +34-981-951364 Fax: +34-981-955100
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Abstract

Endoscopic ultrasound (EUS) has become an essential tool for the study of pancreatic diseases. Specifically, EUS plays a pivotal role evaluating patients with a known or suspected pancreatic mass. In this setting, differential diagnosis remains a clinical challenge. EUS-guided fine-needle aspiration (FNA) and fine-needle biopsy (FNB) have been proven to be safe and useful tools in this setting. EUS-guided FNA and FNB, by obtaining cytological and/or histological samples, are able to diagnose pancreatic lesions with high sensitivity and specificity. In this context, several methodological features, trying to increase the diagnostic yield of EUS-guided FNA and FNB, have been evaluated. In this review, we focus on the role of rapid on-site evaluation (ROSE). From data reported in the literature, ROSE may increase diagnostic yield of EUS-FNA specimens by 10%-30%, and thus, diagnostic accuracy. However, we should point out that many recent studies have reported adequacy rates of > 90% without ROSE, indicating that, perhaps, at high-volume centers, ROSE may not be indispensable to achieve excellent results. The use of ROSE can be considered important during the learning curve of EUS-FNA, and also in hospital with diagnostic accuracy rates < 90%.

Core tip: Endoscopic ultrasound (EUS) has become a crucial tool for the evaluation of solid pancreatic masses. EUS-guided fine-needle aspiration (FNA) and fine-needle biopsy (FNB) have been proven to be safe and useful tools in this setting, and can diagnose pancreatic lesions with high sensitivity and specificity. The use of rapid on-site evaluation can increase adequacy rates and diagnostic yield of EUS-guided FNA or FNB by 10%-30%.

INTRODUCTION

Endoscopic ultrasound (EUS) has become a crucial tool in the study of pancreatic diseases. Specifically, EUS plays a pivotal role when evaluating patients with pancreatic

solid tumors[1,2]. Distinguishing different types of pancreatic solid tumors is an important clinical challenge. Therapeutic strategy in this context is based on the ability to determine the presence of a malignant lesion. Although ductal adenocarcinoma is considered as the main cause of pancreatic mass, many other neoplasms (*e.g.*, lymphoma, cystic tumors, and metastasis) and benign conditions (*e.g.*, chronic pancreatitis) with different prognoses and treatment options can be detected in the pancreas. Taking this into account, a cytopathological confirmation is highly relevant for establishing the best treatment.

EUS-guided fine-needle aspiration (FNA) and fine-needle biopsy (FNB) are considered safe and accurate methods for tissue sampling of intramural and extramural gastrointestinal lesions, including the pancreas. In fact EUS-guided FNA and FNB, by obtaining cytological and/or histological samples, are able to diagnose pancreatic lesions with high sensitivity and specificity[3]. Several studies have evaluated the accuracy of cytology and/or histology after EUS-guided FNA or FNB for the diagnostic evaluation of pancreatic masses.

There are several methodological features, including trying to increase the diagnostic yield of EUS-guided FNA and FNB by rapid on-site evaluation (ROSE). Although experts recommend an on-site evaluation of samples obtained in order to optimize the diagnostic yield of EUS-guided FNA, its effect on diagnostic accuracy has not been properly defined. Reports on the need for ROSE during the procedure are scarce. In this review, we first analyze the role of EUS-guided FNA and FNB for the diagnosis of solid pancreatic masses. Finally, we present the most relevant data published, analyzing the role of EUS-guided FNA and FNB with ROSE in this setting.

USEFULNESS OF EUS-GUIDED FNA AND FNB IN THE DIFFERENTIAL DIAGNOSIS OF SOLID PANCREATIC TUMORS

The role of EUS-guided FNA in the diagnosis of solid pancreatic tumors has been evaluated in many well-designed studies. Reported sensitivity and accuracy for malignancy ranges from 75% to 92% and from 79% to 92%, respectively[4-14]. Two large reviews have been published evaluating the accuracy of EUS-guided FNA in pancreatic masses. One of them included 28 studies (4225 patients). The authors evaluated the usefulness of EUS-FNA to differentiate between benign and malignant lesions. Sensitivity, specificity and diagnostic accuracy were 83% (54%-95%), 100% (71%-100%) and 88% (65%-96%), respectively[15]. The second one, a more recent meta-analysis, published by Hewitt *et al*[16], included 33 studies, with a total of 4984 patients. The authors showed that sensitivity for malignant cytology was 85% (95%CI: 84%-86%), and specificity was 98% (95%CI: 97%-99%). When including atypical and suspicious cytology as true positive, sensitivity increased to 91% (95%CI: 90%-92%); however, the specificity decreased to 94% (95%CI: 93%-96%). EUS-guided FNA also showed a good positive predictive value (99%) and a good negative predictive value (64%). However, it is important to point out that in cases with findings related to chronic pancreatitis, the sensitivity of EUS-guided FNA for the detection of malignancy is clearly decreased[17,18].

In order to optimize tissue retrieval, with the aim of obtaining core specimens, various EUS-guided techniques have been explored. One approach is the use of the Tru-Cut needle (Quick-Core), with variable success and complication rates[19-21]. This Tru-Cut needle has demonstrated that histological samples can be obtained safely[21,22]. However, there are certain limitations with the Quick-Core needle that preclude its routine clinical use. Most importantly, its diagnostic yield is limited for lesions located in the pancreatic head, related to the mechanical friction of the needle-firing mechanism associated with the bent position of the scope[23-25]. In this setting, a novel needle has been designed (Procore) to overcome the limitations of the Tru-Cut needle (mainly in the second portion of the duodenum). A study published with the 19-gauge caliber needle allowed a histological evaluation with an overall accuracy of 85.9% (89.4% in pancreatic solid lesions)[26], with a high inter-observer agreement between pathologists when evaluating the quality of the samples obtained[27]. A new study has been recently published using the 22-gauge Procore needle in pancreatic masses, which was able to obtain a sample suitable for histological evaluation in 88.5% of the cases[28].

However, certain drawbacks of EUS-guided FNA need to be emphasized. In certain cases, the procedure is difficult to perform, because of vessel interposition, duodenal stenosis and/or tumor hardness, particularly in chronic pancreatitis, which hampers the overall accuracy of the procedure. In other occasions, EUS-guided FNA samples cannot be interpreted due to bleeding or noncellular samples. A systematic review of 53 studies estimated a negative predictive value of EUS-guided FNA in the diagnosis of pancreatic adenocarcinoma of 60%-70%[15]. In patients with indeterminate or negative findings at the first EUS-guided FNA, presenting a high suspicion of malignancy, repeating the procedure is highly recommended. Several studies have demonstrated that performing a second EUS-guided FNA was useful for determining the correct and true situation in a high percentage of cases with inconclusive findings at initial EUS-guided FNA; in fact, by repeating EUS-guided FNA up to three times, sensitivity can increase up to 90%[29-31]. Hence, a new puncture seems necessary to exclude malignancy in cases where the first EUS-guided FNA was negative for malignancy. When combining all the information available on the high accuracy in the evaluation of pancreatic tumors, Eloubeidi *et al*[32] recommended performing EUS-guided FNA in all patients with solid pancreatic masses.

ROLE OF ROSE AFTER EUS-GUIDED FNA

The idea of including ROSE is based on the fact that up to 30% of FNA interpretation may be nondiagnostic, because of multiple factors, including scant cellularity and/or crush artifacts from poor slide preparation. In this setting, ROSE of FNA specimens may be beneficial for rapid clinical diagnosis, probably decreasing the number of nondiagnostic procedures. However, data on the role of ROSE are limited, with scant data available over the past few years. In the recently published guidelines from the European Society of Gastrointestinal Endoscopy, the role of ROSE and its relevance in EUS-guided sampling in gastroenterology has been described[33]. We try to analyze different aspects of ROSE evaluation after EUS-guided FNA, and its clinical usefulness in the diagnosis of solid pancreatic masses.

Visual inspection of the samples obtained

It is not clear whether the evaluation of the samples obtained after puncture is useful in order to increase the accuracy of EUS-guided FNA. Neither trained technologists nor cytotechnologists, in a prospective double-blinded study, could properly establish the obtention of an adequate sample by gross visual inspection. The κ score for the agreement between visual evaluation and final microscopic assessment was only 0.2, which is considered poor. False-positive assessments occurred in 30% of the slides[34].

ROSE performed by cytopathologists and cytotechnicians

The role of ROSE has been mainly studied in percutaneous FNA. In this setting, ROSE is accepted as useful, by diminishing the number of inadequate diagnoses. In addition, ROSE may have an impact on costs by decreasing the number of repeat procedures[35-37]. However, data on ROSE in EUS-guided FNA are scarce. Published data suggest that the presence of a cytopathologist during EUS-FNA is cost-effective and useful. We summarize the most important and relevant data reported in the literature.

Chang *et al*[38] reported a 100% rate of adequate specimens with the on-site evaluation of a cytopathologist during EUS-guided FNA. However, the absence of an on-site cytological evaluation resulted in 29% of patients requiring a second procedure to obtain an adequate specimen. Erickson *et al*[39] also published a lower diagnostic accuracy of EUS-FNA (decreasing by 10%-15%) without the presence of an on-site cytopathologist. The only concerns were the prolonged procedure time and the potentially increased risk of complications from the need for multiple needle passes. Klapman *et al*[40] demonstrated that an on-site cytopathologist evaluating the samples improved the diagnostic accuracy of EUS-FNA. In their study, they analyzed the EUS-guided FNA results from two university hospitals. At Center I, 108 patients underwent EUS-guided FNA in the presence of an on-site cytopathologist. At Center II, 87 patients underwent

EUS-guided FNA in the absence of cytopathologist. All procedures at both hospitals were performed by the same endosonographer. At Center I, a definite diagnosis of positive or negative for malignancy was reported in 78% compared with 52% for Center II (OR = 2.94; *P* = 0.001); the rate of patients with an unsatisfactory sample was 9% compared with 20% in Center II (OR = 0.36; *P* = 0.035). Iglesias-Garcia *et al*[14] published their experience in a study including a total of 182 patients. An on-site cytopathologist was available in 95 cases (52.2%). A significantly higher number of needle passes was performed when ROSE was not available (3.5 ± 1.0 *vs* 2.0 ± 0.7; *P* < 0.001). The presence of an on-site cytopathologist was associated with a significantly lower number of inadequate samples (1.0% *vs* 12.6%, *P* = 0.002), and significantly higher diagnostic sensitivity (96.2% *vs* 78.2%; *P* = 0.002) and overall accuracy (96.8% *vs* 86.2%; *P* = 0.013) for malignancy. In a prospective study evaluating 540 patients who underwent EUS-guided FNA procedures of 656 lesions (mostly of pancreatic masses and lymph nodes), which ROSE was available for 607 lesions. From all lesions evaluated on-site by a cytologist, 5/243 considered initially benign (2.1%) finally turned to be malignant. In contrast, among 300 lesions considered malignant after ROSE, 294 (98%) were still malignant at the final report. Agreement was excellent between ROSE and final cytological evaluation (κ = 84.0%, 95%CI: 80.2-87.7). Compared with the true final status, accuracy for final interpretation was slightly higher for ROSE (95.8% *vs* 93.9%). Most of the discrepancies were related the characteristics of the lesions, either because of the presence of scanty cells, strange morphology, or because of the need for different types of immunostaining for final diagnosis[41]. Collins *et al*[42], over a consecutive 3-year period, analyzed 379 patients that underwent ROSE and 377 patients that did not. The percentage of repeat procedures on the non-ROSE group was 5.8%, which was slightly higher than in the ROSE group (2.9%). The use of ROSE decreased the number of repeated procedures by approximately 50% (*P* = 0.024). In patients requiring an additional procedure, the use of ROSE provided a higher number of definitive diagnoses. However, the presence of a cytopathologist is not always possible in many centers, mainly because of the availability according to the organization of the pathology department, which is directly associated with costs. Trying to overcome this common situation, Alsohaibani *et al*[43], in a retrospective study suggested that on-site cytotechnologist interpretation of adequacy of tissue samples might also be useful for improving the diagnostic yield of EUS-guided FNA. The patients were divided into two groups. In Group I, samples were prepared by an endoscopy nurse (*n* = 47) and in Group II by an on-site cytotechnologist (*n* = 55). Pancreatic masses were the main target site. The final diagnosis was higher in the group with on-site cytotechnologists preparing the slides (77% *vs* 53%), suggesting that if an on-site cytopathologist cannot be provided, a trained cytopathology technician should be present to provide an assessment of

Table 1 Diagnostic performance of rapid on-site evaluation by a cytopathologist/cytotechnician in the evaluation of solid pancreatic masses

Ref.	Year	No. of cases		Accuracy		
		With ROSE	Without ROSE	With ROSE	Without ROSE	*P* value
Klapman *et al*[40]	2003	108	87	78%	52%	0.001
Alsohaibani *et al*[43]	2009	47	60	77%	53%	0.001
Iglesias-Garcia *et al*[14]	2011	95	987	96.80%	86.20%	0.013
Collins *et al*[42]	2013	379	377	97.10%	94.10%	N.S.

ROSE: Rapid on-site evaluation.

sample adequacy.

However, ROSE was not found to be better than the standard approach in all studies. In a prospective multi-center study with 409 patients, two centers used ROSE and two did not[8]. Results were similar in both groups, and merely differed in a higher negative predictive value in the subgroup of patients with extraintestinal mass lesions in the group with ROSE. In another study (analyzing 247 pancreatic solid lesions and 276 lymph nodes), a retrospective analysis of risk factors for inadequate EUS-guided FNA specimens was performed. Cytopathological adequacy was higher for lymph nodes (96% *vs* 84%, *P* = 0.008) but not for pancreatic solid lesions (99% *vs* 100%; *P* = 1) if ROSE was available[44].

Finally, a recent meta-analysis aimed to determine whether ROSE, together with the variability of the reference standard and other sources of heterogeneity may affect the diagnostic yield of EUS-guided FNA when evaluating solid pancreatic masses[45]. Hebert-Magee *et al*[45] included 34 studies. The pooled sensitivity and specificity was 88.6% (95%CI: 87.2%-89.9%) and 99.3% (95%CI: 98.7%-99.7%), respectively. The LR+ and LR- were 33.46 (95%CI: 20.76-53.91) and 0.11 (95%CI: 0.08-0.16), respectively. In this study, the main factor determining the accuracy of EUS-guided FNA was the presence of ROSE (*P* = 0.001). Thus, EUS-guided FNA was considered an effective modality in the diagnosis of pancreatic cancer when evaluating solid pancreatic lesions, which was higher with the availability of ROSE.

Another important point is the potential application of ROSE with the use of new histological needles. A recent study from Krishnan *et al*[46] aimed to investigate the utility of ROSE in achieving a final diagnosis for EUS-guided FNB core specimens. The authors evaluated 60 consecutive patients referred for EUS-guided FNA of lesions inside or adjacent to the gastrointestinal tract. All patients underwent EUS-guided FNB to evaluate the additive value of ROSE to the diagnostic accuracy of specimens obtained using a core biopsy needle. EUS-guided FNB was feasible in all 60 cases. On-site specimen adequacy and final diagnostic accuracy was 58% (95%CI: 45.1%-71.2%) and 83% (95%CI: 71.9%-91.5%), respectively. Results were better than those obtained for standard EUS-guided FNA.

Table 1 summarizes the diagnostic accuracy from the most relevant papers comparing EUS-guided FNA or FNB with and without ROSE.

However, little is known about the impact of ROSE on EUS-guided FNA procedural time, and it remains unclear whether using ROSE prolongs the procedure or makes it less time-consuming by reducing the number of needle passes. According to some published data, it is assumed that an average time for obtaining the specimen and performing on-site examination is 15 min per sample[47]. Average time used by the cytopathologist for ROSE in computed-tomography-guided and ultrasound-guided FNA specimens is relatively high (48.7 and 44.4 min, respectively)[48].

Regarding complications, scarce information is available from the different studies. In the study from Iglesias-Garcia *et al*[14], complication rate in the group of cases without ROSE was significantly higher, probably related to the higher number of passes needed to obtain the final diagnosis. However, all complications reported were considered as mild, and no mortality was associated with the procedures.

ROSE performed by an endosonographer

As previously commented, the presence of cytopathologist is not possible in all centers, for all EUS-guided FNA or FNB procedures. In this context, there is a trend to train endosonographers for ROSE during EUS-guided FNA, in order to reduce costs. Some studies have attempted to resolve this question.

A prospective double-blind study showed that even experienced endosonographers, trained in the management of samples obtained by FNA, were less accurate than a cytotechnician in assessing specimen adequacy (68%-76% *vs* 82%; *P* = 0.004) and in the determination of malignancy (69%-72% *vs* 89%; *P* < 0.001)[49]. A second study, including 73 procedures, could not find any difference when analyzing sample adequacy, number of needle passes, or EUS-guided FNA performance characteristics in two different 2-year periods. In one of them, ROSE was performed by endosonographers and in the other, this evaluation was performed by cytopathologists[50]. Hayashi *et al*[51] retrospectively evaluated patients from two different periods who underwent EUS-guided FNA for the study of solid pancreatic masses. Before initiating ROSE at the start of the second period, two endosonographers underwent training for cytological interpretation, focused on four cytological features of pancreatic ductal

carcinoma: anisonucleosis, nuclear membrane irregularity, overlapping, and enlargement. During EUS-guided FNA in Period 2, endosonographers classified the Diff-Quik smears under three atypical grades and evaluated the adequacy. One made all diagnoses. The rate of inconclusive diagnoses, interpreted as suspicious, atypical, and inadequate for diagnosis was reduced from 26.4% to 8.2% (P = 0.004). Moreover, diagnostic accuracy increased from 69.2% to 91.8% (P < 0.001). Authors concluded that samples evaluated by trained endosonographers, with a simple cytological grading system, could be considered useful in this context.

CONCLUSION

EUS-guided FNA and FNB are effective modalities for the diagnosis of solid pancreatic masses, with high diagnostic accuracy. It is well known that diagnostic performance is clearly associated with the presence of a skilled team, including both endosonographers and cytopathologists. In this context, ROSE appears to be a useful tool for optimizing the yield of this procedure. Although gross visual inspection cannot assess the adequacy of EUS-guided FNA or FNB specimens for cytopathological examination, ROSE performed by cytopathologists provides a highly accurate diagnosis with an excellent agreement with the final cytopathological diagnosis. ROSE may increase adequacy rates of EUS-guided FNA or FNB specimens by 10%-30%. However, we should point out that many recent studies have reported adequacy rates > 90% without the use of ROSE, indicating that, in high-volume centers, ROSE may not be indispensable to achieve excellent results. Finally, data on cost-effectiveness are limited. After analyzing all data available, implementation of ROSE should be considered, mainly for the learning curve of the technique and at centers in which specimen adequacy rates are < 90%.

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2014 ADVANCES IN PANCREATIC CANCER

Molecular pathology of intraductal papillary mucinous neoplasms of the pancreas

Marina Paini, Stefano Crippa, Stefano Partelli, Filippo Scopelliti, Domenico Tamburrino, Andrea Baldoni, Massimo Falconi

Marina Paini, Domenico Tamburrino, Department of Surgery, University of Verona, 37134 Verona (VR), Italy

Stefano Crippa, Stefano Partelli, Andrea Baldoni, Massimo Falconi, Division of Pancreatic Surgery, Ospedali Riuniti, Università Politecnica delle Marche, 60126 Ancona (AN), Italy

Filippo Scopelliti, Division of Pancreatic Surgery, Casa di Cura Dott. Pederzoli, 37019 Peschiera del Garda (VR), Italy

Author contributions: Paini M and Crippa S contributed equally to the paper; Paini M and Crippa S designed the research; Paini M, Crippa S, Partelli S, Scopelliti F, Tamburrino D and Baldoni A reviewed the literature and performed the review; Paini M and Crippa S wrote the paper; Falconi M reviewed all the work.

Correspondence to: Massimo Falconi, MD, Chief, Division of Pancreatic Surgery, Ospedali Riuniti, Università Politecnica delle Marche, Via Conca 71, 60126 Ancona (AN), Italy. m.falconi@univpm.it

Telephone: +39-71-5965781   Fax: +39-71-5964429

Abstract

Since the first description of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas in the eighties, their identification has dramatically increased in the last decades, hand to hand with the improvements in diagnostic imaging and sampling techniques for the study of pancreatic diseases. However, the heterogeneity of IPMNs and their malignant potential make difficult the management of these lesions. The objective of this review is to identify the molecular characteristics of IPMNs in order to recognize potential markers for the discrimination of more aggressive IPMNs requiring surgical resection from benign IPMNs that could be observed. We briefly summarize recent research findings on the genetics and epigenetics of intraductal papillary mucinous neoplasms, identifying some genes, molecular mechanisms and cellular signaling pathways correlated to the pathogenesis of IPMNs and their progression to malignancy. The knowledge of molecular biology of IPMNs has impressively developed over the last few years. A great amount of genes functioning as oncogenes or tumor suppressor genes have been identified, in pancreatic juice or in blood or in the samples from the pancreatic resections, but further researches are required to use these informations for clinical intent, in order to better define the natural history of these diseases and to improve their management.

Core tip: The heterogeneity and the malignant potential of intraductal papillary mucinous neoplasms make their management still controversial. The identification of potential markers correlated to the pathogenesis of intraductal papillary mucinous neoplasms and with their progression to malignancy could be useful to discriminate lesions requiring surgical resection from benign neoplasms that could be followed-up.

INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMNs) are

one of the most fascinating pancreatic neoplasm to be characterized in the last few decades. Described for the first time by Ohashi *et al*[1] in 1982 as a separate tumor from mucinous cystic neoplasms (MCNs) and pancreatic ductal adenocarcinoma (PDA), they were confused or misdiagnosed with MCNs for many years. It is now clear that these two mucin-producing neoplasms are clearly separate entities. MCNs are characteristically defined by an ovarian-like stroma, they do not arise from the ductal system of the pancreas and they are single lesions located almost exclusively in the pancreatic body-tail of middle-aged women[2,3]. IPMNs are mucin-producing neoplasms, arising from the native pancreatic ducts with prominent intraductal growth and frequent papillary architecture[4]. Although the true incidence of IPMNs is unknown, these neoplasms are nowadays frequently recognized because of the widespread use of cross-sectional imaging and because of the greater awareness of this entity among radiologists, gastroenterologists and surgeons[5,6]. Data from high-volume centers for pancreatic surgery suggest that about 15% to 20% of all pancreatectomies are performed for IPMN[7]. Interestingly, in an autopsy study, small cystic lesions possibly representing IPMNs were found in 24% of elderly patients[8]. As our understanding of IPMNs has grown over time, it has become clear that IPMNs are a heterogeneous group of tumors, with different clinical and radiological presentation, different risk of malignancy and different management. In this light, it is important the preoperative prediction of the malignant potential of an IPMN in order to balance the potential complications of pancreatic surgery with the potential risk of being or become malignant over time. Hence, for a better and complete decision making, it is important to consider also the molecular pathology of IPMNs to identify molecular markers of "high-risk" lesions. Nevertheless, the information we have about molecular mechanisms involved in their carcinogenesis is still poor. The aim of this review is to present the role of the oncogenic and the tumor suppressor pathways in the neoplastic transformation of IPMNs.

CLASSIFICATION OF IPMNS

IPMNs can be classified according to the involvement of pancreatic ductal system in[4,9]: (1) main-duct type, when the tumor involves only the main pancreatic duct; (2) branch-duct type, when the tumor involves only branch-ducts, with no macroscopic or microscopic involvement of the main pancreatic duct; and (3) combined type, when the neoplasms involved macroscopically and/or microscopically both the main pancreatic duct and its side branches.

PATHOLOGY OF IPMNS

A clear identification and distinction of main-duct, combined and branch-duct IPMNs is not only of taxonomic significance, but has a practical impact on patient man-agement. Combined IPMNs show close overlapping similarities with main-duct IPMNs in regard to clinico-pathological and epidemiological characteristics[6]. Moreover, branch-duct IPMNs have a lower risk of malignant degeneration compared to main-duct/combined IPMNs and non-surgical management is feasible for many of these lesions, thus avoiding "prophylactic" pancreatec-tomy with its associated risks[9-12]. Histologically, IPMNs can be categorized in four groups based on the degree of cytoarchitectural dysplasia[13,14]: (1) IPMN with low-grade dysplasia (IPMN adenoma); (2) IPMN with mod-erate intermediate-grade dysplasia (IPMN borderline); (3) IPMN with high-grade dysplasia (IPMN with carcinoma *in situ*); and (4) IPMN with invasive carcinoma (invasive IPMN).

Remarkably, IPMNs may show different degrees of dysplasia within the same lesions. As colonic adenomas, they can show neoplastic transformation culminating in invasive carcinoma. Approximately 15%-20% of branch-duct IPMNs and 40%-50% of main-duct IPMNs harbor an invasive carcinoma[9]. In half of the cases, the inva-sive carcinoma has a colloid (or muconodular) pattern of invasion, while in the remaining 50% of the cases a tubular (or conventional) ductal pattern is present[14,15]. Tubular-type is morphologically indistinguishable from ordinary PDA. Colloid type is very similar to colloid carcinoma of the breast and it is associated with a good prognosis[15]. Chemotherapy for patients with resected invasive IPMN is recommended[16]; adjuvant treatment is advisable also in case of positive resection margins or lymph node metastases[17]. Moreover, lifetime surveil-lance is required also for non-invasive IPMN after par-tial pancreatectomy since the risk of local recurrence in the pancreatic remnant is about 8%-10% and it may be the expression of a new metachrous tumor. The neo-plastic epithelial cells in an IPMN can have a variety of directions of differentiation[18]. Histological subtypes of IPMNs include: (1) intestinal type: morphologically it is very similar to colonic villous adenomas. Most main-duct IPMNs are of intestinal type, carrying a higher risk of invasive carcinoma, more commonly of the colloid type; (2) gastric type: virtually indistinguishable from gastric mucosa, it is characterized by a low proliferative activity with a very low risk of malignant transformation. It is the most common subtypes among branch-duct IPMNs; (3) pancreatobiliary type: it is characterized by a complex papillary configuration and it is uncommon. Usually it is associated with at least high-grade dysplasia and it is considered as the high-grade version of gastric type. When invasive carcinoma is present, it is frequently of the tubular type; and (4) oncocytic type: originally de-scribed as a separate entity[19], this IPMN subtype shows proliferative cells associated with atypical cytology. It is associated with high-grade dysplasia.

Finally, multifocal lesions can be frequently found in IPMNs. Main-duct IPMNs may involve the entire main pancreatic duct or "skip" lesions can occur with synchro-nous, multifocal involvement of main duct epithelium[20].

Multifocal IPMNs are frequently found in the branch-duct type and they represent a challenging situation both for IPMNs undergoing surgical resection and for those which are non-operatively managed. The presence of multifocal branch-duct IPMNs does not seem to be associated with an increased risk of malignancy[21].

ONCOGENES AND INTRADUCTAL PAPILLARY MUCINOUS NEOPLASMS

Kirsten ras oncogene
Kirsten-ras (KRAS) is located on chromosome arm 12p and encodes a membrane-bound guanosine triphosphate (GTP)-binding protein. In some cases the mutation can occur at the codon 13[22]. The frequency of the KRAS gene mutations in IPMNs varies from study to study, ranging from 38.2%[23] to 100%[24-26]. The wide variety of the reported frequencies is most likely due to the ongoing better definition of these lesions[13,18,27] and might also be dependent on the sensitivity of a chosen screening methodology[28]. There is no significant difference among the incidence of KRAS mutation in the various grades of dysplasia: 87% in low-grade, 90.2% in intermediate grade and 70.7% in high-grade dysplasia[29]; anyway, this mutation is considered to be an early event in the neoplastic transformation of IPMNs[30].

The expression of KRAS mutation can be found both in surgically resected specimens, in the peripheral blood[31] and in the pancreatic juice; however, in the latter case, KRAS mutations are not a specific marker for pancreatic neoplasms because similar mutations were detected in the pancreatic juice from patients with chronic pancreatitis or other pancreatic diseases[32,33]. Moreover KRAS mutation is frequently found also in the peritumoral region and in other lesions of IPMN, but only if the mutation is present in the main tumor[30].

Furthermore, a significant difference in the diameter of the main pancreatic duct between patients with and without the mutant *KRAS* gene was detected; this suggests that the incidence of KRAS mutation may be associated with the hypersecretion of mucin[34]. KRAS mutations have the highest frequency in the pancreatobiliary subtype (100%) and the lowest frequency in the intestinal subtype (46.2%)[29].

GNAS complex locus
GNAS is an oncogene located on the long arm of chromosome 20 at position 13.3, encoding the guanine nucleotide-binding protein (G-protein) alpha subunit (G_s-α)[35]. Mutations at codon 201 were detected in 61% of IPMNs[36]. An important aspect for the differential diagnosis of pancreatic cystic lesions is that GNAS mutations were not found in cystic neoplasms other than IPMN or in invasive adenocarcinomas not associated with IPMN[29]. The prevalence of GNAS mutations is higher in more advanced lesions, from 19% in low-grade dysplasia, to 23% in intermediate grade and 58% in high-grade dysplasia. As regarding histological subtypes, 100% of the

intestinal type harbored GNAS mutations, while 71% of pancreatobiliary type and 51% of gastric type shows mutant GNAS; in oncocytic IPMNs no mutation of GNAS is found[36].

"PI3K/Akt" signaling pathway
Phosphatidylinositol-3 kinases (PI3Ks) constitute a large and complex family of lipid kinases encompassing three classes with multiple subunits and isoforms[37]. They play an important role in several cellular functions, such as proliferation, differentiation, chemotaxis, survival, trafficking and glucose homeostasis[37]. Phosphoinositide-3-kinase-catalyc-alfa gene (*PI3KCA*) mutations frequently occur in exon 9 and 20 in more of 75% of the cases, affecting the functionally important helical and kinase domains of the protein[38-40]. Particularly, the mutations E542K, E545K and H1047R seem to elevate the lipid kinase activity of *PIK3CA* and to activate the Akt signaling pathway. In IPMNs, the frequency of the somatic mutation of the *PIK3CA* gene is 11%[28]. *PIK3CA* mutation seems to be a rather late event in the transition of these lesions to malignancy[28,41]. In PDA cell lines no mutation is found in the entire coding region of the *PIK3CA* gene[38].

BRAF and the RAS/MAPK pathway
B-Raf is a serine/threonin kinase, encoded by BRAF, located immediately downstream in Ras signaling, in the RAS/MAPK pathway that regulates proliferation, differentiation, migration and apoptosis of the cells. The Ras pathway involves the kinase cascade Raf/MEK/ERK; BRAF is one of the three isoforms that Raf shows (the others are a-Raf and c-RAF/Raf-1)[22].

The *BRAF* gene is located on the long arm of chromosome 7 at position 34. The rate of the somatic BRAF mutation described for IPMN is only 2.7%[22,28]. Even if the BRAF mutation is not frequent, the alteration of the Ras-Raf-MEK-ERK-MAP kinase pathway by BRAF mutation together with Ras mutation may play an important role in the tumorigenesis of IPMNs[22,28]; it is possible that tumors with both BRAF and KRAS mutations have an accelerated course in the development or progression[28].

Telomerase reverse transcriptase expression
Human telomeres are nucleoprotein complexes in the chromosomes ends, essential to genomic stability[42]. When telomeres become critically short, after repeated replication-dependent loss of DNA termini[43] during various cell divisions, loss their capping function and so, protective mechanisms avoid genetic instability, eliminating senescent cells[42]. However, in case of activation of telomerase, the function of telomeres can be restored through the elongation of telomeric DNA sequences and the overcoming of the *telomere crisis*[44], thus the cells survive with accumulation of multiple genomic and epigenetic aberrations[45]. Telomerase is a RNA-dependent DNA polymerase, generally inactivated in normal human

Table 1 Most commonly oncogenes genetically altered in intraductal papillary mucinous neoplasms						
Ref.	Gene symbol	Gene name	Mechanism of genetic alteration	Chromosome site	Known or predicted function	Alteration in IPMN
Z'graggen *et al*[24], 1997	*KRAS2*	v-Ki-ras-2 Kirsten rat sarcoma viral oncogene homolog	Point mutation	12p12.1	Signal transduction, proliferation, cell survival, motility	81.2%
Dal Molin *et al*[36], 2013	*GNAS*	GNAS complex locus	Point mutation	20q13.3	G-protein beta/gamma-subunit complex binding	61%
Schönleben *et al*[41], 2006	*PI3KCA*	Phosphoinositide-3-kinase	Point mutation	3q26.3	Proliferation, differentation, chemotaxis, survival, trafficking, glucose homeostasis	11%
Schönleben *et al*[22], 2007	*BRAF*	v-raf murine sarcoma viral oncogene homolog B1	Point mutation	7q34	Signal transduction, cell growth	2.7%
Hashimoto *et al*[48], 2008	*TERT*	Telomerase reverse transcriptase	Deletion, nonsynonymous mutation, polymorphisms	5p15.3	Genome stability	70.6%
Jang *et al*[50], 2007	*SHH*	Sonic hedgehog	Point mutation, missense mutation	7q36	Cell growth, cell specialization, proliferation of adult stem cells	61.8%

IPMN: Intraductal papillary mucinous neoplasms.

somatic cells. Its catalytic component is encoded by the human telomerase reverse transcriptase *(hTERT)* gene, located on chromosome 5[46,47]. In IPMNs, it is possible to notice a gradual decrease of the telomeres with histologic progression; they are shortened in the 97.3% of the loci analyzed[48]. Telomere shortening can be observed in any case of adenoma, with a 50% reduction in length in half of the foci examined[48]. As in carcinoma *in situ* IPMNs the telomere shortening is significant compared to borderline IPMNs, but non compared to PDAs, it is likely that telomeres shorten to a critical length at the carcinoma *in situ* histological grade[48]. Telomerase activity is significantly higher in invasive carcinoma than in borderline or carcinoma *in situ*. In malignant IPMNs, also hTERT expression is higher than in non-malignant neoplasms: 15.8% in adenoma, 35.7% in borderline, 85% in carcinoma *in situ* and 86.7% in invasive carcinoma[48]. Then, transition from borderline to carcinoma *in situ* seems to be the critical step at which telomere dysfunction occurrs during IPMN carcinogenesis; the ability of the cells to overcome this crisis through the up-regulation of hTERT is a promoting event for the development of malignancy[44,45,48].

Hedgehog signaling pathway
The hedgehog family consists of three homologous genes: the first two discovered, desert hedgehog (Dhh) and Indian hedgehog (Ihh), were named after species of the spiny mammal, whereas the third, sonic hedgehog (Shh), was named after the character from the popular Sega Genesis video game. Hedgehog (Hh) proteins are a family of secreted signaling factors that regulate the development of many organs and tissues[49]. Shh is the best studied ligand of the Hedgehog signaling pathway; abnormal activation of Hedgehog pathway is described in IPMNs: Shh expression is detected in 46.2% of adenomas, 35.7% of borderline dysplasia IPMNs, 80% of

carcinoma *in situ*-IPMNs and 84.6% of invasive carcinomas with a significant increase in malignant IPMNs[50]. Evaluating IPMN by histological subtype, Shh is expressed in 68.8% of intestinal types, in 92.8% of pancreatobiliary types, 38.1% of null types and in 50% of unclassifiable types[50]. Shh expression is detected also in stromal cells, rarely in adenoma and borderline IPMNs, significantly in malignant IPMNs[50]. In addiction, its expression is showed in tumor cells of metastatic lymph nodes[50].

As Shh expression is detectable in pancreatic juice from IPMN, but it is absent in pancreatitis juice, Shh measurement in pancreatic juice could discriminate IPMN from chronic pancreatitis[51].

These data suggest that the activation of the Shh signaling pathway may be involved in an early stage of the development of IPMN[50-52] and contributes to the transformation from benign to malignant dysplasia in IPMN[52], especially in the intestinal and pancreatobiliary types. Shh expression may also have a role in metastatic progression to lymph node in malignant IPMN[50]. Oncogenes and intraductal papillary mucinous neoplasms show in Table 1.

TUMOR SUPPRESSOR GENES AND INTRADUCTAL PAPILLARY MUCINOUS NEOPLASMS

Cyclin-dependent kinase inhibitor 2A/p16
CDKN2A is a tumor suppressor gene on chromosome 9p21 (the locus of Ink4A) that encodes the cyclin dependent kinase (Cdk) inhibitor p16[Ink4A]. This protein inhibits progression of the cell cycle at the G1-S checkpoint (under the control of the Retinoblastoma pathway), binding to cyclin-dependant kinases (CDKs)[53] and provoking Retinoblastoma protein phosphorylation:

its loss of function results in the acceleration of cell growth[34,54]. Different mechanisms are responsible of its inactivation, such as homozygous deletion, intragenic mutation and epigenetic silencing by promoter methylation. All of these invalidating modifications arise at late stages of pancreatic carcinogenesis. While the loss of p16 protein described in adenoma/borderline IPMNs varies from 10%[55] to 25%[56], in the IPMN carcinomas the inactivation of the suppressor gene is found in 77.8%[56]-100%[55] of the cases. Observing all the degrees of histological atypia, loss of heterozygosity of the *p16* gene was described increasingly from adenoma (12.5%) to IPMNs with intermediate dysplasia (20%), high-grade dysplasia (33%) and invasive carcinoma (100%)[26]. It's evident that p16 loss is necessary but not sufficient to induce progression from non-invasive IPMN to invasive carcinoma; in any case, loss of p16 alone is considered the strongest marker in differentiating IPMN with low-grade/intermediate dysplasia from the IPMN with carcinoma (*in situ* or invasive)[55].

TP53 gene

The tumor suppressor gene *p53* is located on the short arm of chromosome 17p; it regulates an essential growth checkpoint that both protects against genomic rearrangement or accumulation of mutations and suppresses cellular transformation caused by oncogene activation or by the loss of tumor suppressor pathways[57]. The roles of p53 protein are the maintenance of G2-M arrest, the regulation of G1-S checkpoint, the induction of apoptosis and the regulation of senescence, the repair of DNA damages and the change of cellular metabolism[58,59]. *p53* gene is inactivated especially by missense mutations in sequences coding for the DNA binding domain[57], through intragenic mutations of allele 1, accompanied with loss of the other allele[60,61]. Even if the percentage of mutated p53 in IPMN is slightly different in the reports, from 27.3%[26] until 42% (in pancreatic juice and in tissue specimens)[62] or 52%[63], the consensus is that in the majority of the cases no mutation of p53 is commonly found in adenoma dysplasia; p53 mutation is detectable in 33.3% of the borderline tumor and in 38.5% of non-invasive carcinoma[64], while in almost all invasive carcinoma *p53* gene is inactivated[26,62,65]. Therefore, loss of heterozygosity (LOH) of *p53* gene seems to be a relatively late event in the carcinogenesis of IPMN, similarly to what happens in colorectal neoplasms[26,66].

In IPMNs, LOH of the *p53* gene concomitant with LOH of the *p16* gene are detected in 0% of the adenomas, in 20% of the borderline tumors, in 33% of the non-invasive carcinomas and in all the invasive carcinomas[26,56]; hence, LOH of p16 and p53, and the accumulation of genetic alterations, are crucial events in the evolution of IPMN to an invasive carcinoma[26].

Deleted in pancreatic cancer locus 4

Deleted in pancreatic cancer locus 4 (DPC4) tumor-suppressor gene, located at chromosomal region 18q21, is involved in the transforming growth factor β (TGF-β) growth inhibitory pathway. DPC4 encodes SMAD4, a nuclear transcription factor that activates transcription of cell cycle inhibitory factors, particularly p21CIP1[58]. DPC4 is inactivated by homozygous deletion and by intragenic mutations accompanied by loss of the other allele[58,67,68]. As a consequence, loss of function of SMAD4 lead to upregulation of the Retinoblastoma pathway with the consequent progression from G1 to S phase of the cell cycle until the unregulated cellular proliferation[54,55,58]. Inactivation of DPC4 is relatively specific for pancreatic adenocarcinoma (inactivated in the 55% of the cases)[67,69,70]. SMAD4 expression is detected in all adenoma/borderline IPMNs and carcinoma *in situ* IPMNs, while it is lost in 75% of invasive carcinoma IPMNs[55]. Then, loss of SMAD4 expression is the best marker for the presence of invasive carcinoma[55]. Among invasive carcinomas, all colloid carcinomas seem to show strong and diffuse positive labeling for DPC4, whereas only 50% of tubular carcinomas present intense DPC4 staining[71]. SMAD4 mutations have also recently been associated with poor prognosis and with the development of widespread metastatic disease in pancreatic cancer[72,73].

Serine/threonine kinase 11 gene

Serine/threonine kinase 11 gene (*STK11*) gene, also known as liver kinase B1 (LBK1), on chromosome arm 19p, encodes a serine/threonine kinase with growth-suppressing activity[74]. Furthermore, the product of STK11/LBK1, in association with p53, regulates p53-dependent apoptosis[75]. Inherited mutations in this gene are responsible for most cases of Peutz-Jegher syndrome (PJS) and they are associated with IPMN and PDA[58]. The biallelic inactivation of the *STK11/LKB1* gene in IPMN of the patients with PJS suggests a causative relationship between the mutation and IPMN[76,77]. However, in addition to germline mutations, somatic mutations can be found in 5% of patients with sporadic IPMN and pancreatic cancers[76,77]. LOH at 19p13.3 is present in 100% of IPMNs from patients with PJS and in 25% from patients lacking features of PJS[77]. As patients with PJS have a 132-fold increased risk of developing pancreatic cancer (that may develop as an IPMN)[78,79], IPMN can be a "curable precursor" of PDA in some of these patients[79,80].

Brahma-related gene 1

BRG1, encoded by the gene at chromosomal region 19p13.2, is an ATPase/helicase and constitutes the catalytic subunit of the SWI/SNF chromatin remodeling complex, that disrupts the adhesion of histone components and DNA, allowing transcription factors access to their target genes. The tumor suppressive nature of BRG1 has been demonstrated in IPMNs, where the loss of BRG1 expression occurs in 53.3% of the lesions, increasing from the low-grade IPMNs (28%) to intermediate-grade (52%) and to high-grade IPMNs (76%); a progressive loss of BRG1 expression is therefore associated with increasing degree of dysplasia[81]. No significant

Table 2 Most commonly tumor suppressor genes genetically altered in intraductal papillary mucinous neoplasms

Ref.	Gene symbol	Gene name	Mechanism of genetic alteration	Chromosome site	Known or predicted function	Alteration in IPMN
Wada *et al*[26], 2002	CDKN2A/ p16	Cyclin-dependent kinase inhibitor 2A	Homozygous deletion (41%), intragenic mutation (38%)	9p21	Cyclin-dependent kinase inhibitor	38.1%
Wada *et al*[26], 2002	TP53	Tumor protein p53	Intragenic mutation in 1 allele and loss in the other allele	17p13.1	Cell cycle arrest, apoptosis, senescence, DNA repair, metabolism change	27.3%
Biankin *et al*[55], 2002	SMAD4/ DPC4	Mothers against decapentaplegic homolog of 4, drosophila	Homozygous deletion (50%), intragenic mutation in 1 allele and loss in the other allele (50%)	18q21.1	Signal transmission	16.7%
Sato *et al*[77], 2001	STK11/ LKB1	Serine/threonine kinase 11	Homozygous deletion, intragenic mutation in 1 allele and loss in the other allele	19p13.3	Apoptosis regulation	32% (100% in patients with PJS and 25% in patients without PJS)
Dal Molin *et al*[81], 2012	BRG1	Brahma-related gene-1	Homozygous deletion, intragenic mutation in 1 allele and loss in the other allele	19p13.2	Regulation of cellular proliferation, regulation of several genes involved in key steps in tumorigenesis	53.3%

IPMN: Intraductal papillary mucinous neoplasm; PJS: Peutz-jeghers syndrome.

correlation between BRG1 expression and the various histological subtypes or the different locations within the ductal system of the cyst has been found[81].

Tumor suppressor genes and intraductal papillary mucinous neoplasms are shown in Table 2.

DNA METHYLATION IN THE TUMOR SUPPRESSOR GENES

DNA methylation of tumor suppressor gene promoter sites is an epigenetic mechanisms that has been demonstrated to play a role in tumorigenesis[82].

Aberrant hypermethylation of gene promoter regions and subsequent loss of gene expression can be observed in several human neoplasms[83-85]; in particular, aberrant methylation involving at least one gene promoter site is present in 92% of the neoplastic cases[82]. As regarding IPMN, silencing of tumor suppressor gene expression by promoter hypermethylation at cytosine-phospho-guanine (CpG) islands (genomic regions that contain a high frequency of CpG sites, found in about 40% of promoters of mammalian genes) is detectable in more than 80% of samples[86].

Aberrant methylation is present in promoter regions of genes with well-characterized roles in tumor suppression, most frequently of genes implicated in cell cycle control, as p16, APC and p73[82].

Significantly, higher grade IPMNs have more methylated genes than lower grade IPMNs[87]; for instance, IPMNs harbor APC hypermethylation in 10% of non-invasive samples and in 50% of the infiltrative ones[82]. A similar difference is detectable in E-cadherin methylation, the gene related to tissue infiltration and locoregional metastasis (10% in non-invasive IPMNs and 38% in invasive IPMNs)[82]. Also the mismatch repair genes *hMLH1* and *MGMT* are more frequently methylated

in the invasive IPMNs than in the non-invasive tumors (38% *vs* 10% and 45% *vs* 20%, respectively)[82]. A significant difference in the aberrant methylation is present in TFPI-2: 85% in high-grade IPMNs and 17% in low-grade IPMNs[88]. Other genes are significantly more likely to be methylated in IPMNs with high-grade than in those with low-grade dysplasia: BNIP3 (57% in IPMNs with high-grade dysplasia, 20% in intermediate-grade dysplasia and 0% in low-grade dysplasia and in normal pancreas samples)[87], PTCHD2 (50% in high-grade dysplasia, 27% in intermediate-grade dysplasia, 0% in low-grade dysplasia and 6% in normal pancreas samples)[87], SOX17, NXPH1, EBF3[87], ppENK and p16[86].

Whereas in the IPMN-associated adenocarcinomas 55% of the samples is methylated at three or more gene promoters, in non-invasive IPMNs the hypermethylation involving three or more promoter regions is detectable in only 20% of the samples (all of these are associated with carcinoma *in situ*)[82].

Hence, the analysis of methylated DNA in pancreatic juice could be useful to differentiate, before surgery, invasive IPMNs from non-invasive IPMNs[86].

Moreover, since methylation at a specific genomic sites may predispose to tumor recurrence also after complete surgical extirpation, the methylation profile of the surgical resection margin in the absence of histological disease could be useful to identify a remnant pancreas at risk for tumor recurrence or for multifocal disease[86].

A significant difference in the percentage of genes methylated is also detectable between main-duct IPMNs and branch-duct IPMNs (71.3% ± 23.8% *vs* 44.4% ± 20%)[87,88].

S100 PROTEIN FAMILY

The members of the S100 family are proteins containing a functional EF-hand calcium-binding domain[89]. They

Table 3 Correlation between mutation of the gene and grade of dysplasia of intraductal papillary mucinous neoplasm

Ref.	Gene	Adenoma	Borderline	Carcinoma *in situ*	Invasive carcinoma
Wu *et al*[29], 2011	KRAS2	87%	90.2%	70.7%	70.7%
Dal Molin *et al*[36], 2013	GNAS	19%	23%	58%	58%
Schönleben *et al*[41], 2006	PI3KCA	0%	2.7%	2.7%	5.5%
Schönleben *et al*[22], 2007	BRAF	0%	0%	0%	2.7%
Hashimoto *et al*[48], 2008	hTERT	15.8%	35.7%	85%	86.7%
Jang *et al*[50], 2007	Shh	46.2%	35.7%	80%	84.6%
Wada[26], 2002	CDKN2A/p16	12.5%	20%	33%	100%
Abe *et al*[64], 2007; Wada *et al*[26], 2002	p53	0%	33.3%	38.5%	100%
Biankin *et al*[55], 2002	DPC4	0%	0%	38%	75%
Dal Molin *et al*[81], 2012	BRG1	28%	52%	76%	76%
Jang *et al*[23], 2009	S100A4	7.4%	7.4%	42.9%	42.9%

are localized in the cytoplasm and/or nucleus of many various cells and they are involved in Ca^{2+} signaling network, cell growth and motility, cell cycle progression, transcription and cell differentiation[90,91]. Sixteen S100 genes are described and they are located as a cluster on chromosome 1q21. Differently, the S100P gene is located at 4p16; it encodes the protein S100P (S100 calcium binding protein P), that, in addiction to binding Ca^{2+}, also binds Zn^{2+} and Mg^{2+}. The expression of this gene is increased in pancreatic cancer[92] and it is involved in its cell growth, survival and invasion[93]. In IPMNs, the levels of S100P in bulk pancreatic tissues are higher than in non-neoplastic pancreatic tissues; in microdissected cells, the levels of the protein are higher than in PDA and in PanIN cells, while, in pancreatic juice, IPMNs express higher levels of S100P than it was detected in chronic pancreatitis[94]. Then, S100P expression in pancreatic juice may be measured to discriminate neoplastic disease from chronic pancreatitis and S100P may be considered an early developmental marker of pancreatic carcinogenesis[94].

Protein S100 calcium binding protein A4 (S100A4), encoded by the *S100A4* gene, is involved in the regulation of the motility and invasiveness of cancer cells and its altered expression is implicated in tumor metastasis[95]. The expression of S100A4 is increasing with the malignancy of IPMN: S100A4 protein is detectable in 7.4% of benign IPMNs (adenoma and borderline dysplasia) and in 42.9% of IPMN carcinomas. Thus, the expression of S100A4 could be a possible marker of malignancy, useful for the diagnosis and for providing the biological behavior of IPMN[23].

Protein S100 calcium binding protein A6 (S100A6), encoded by the *S100A6* gene, is implicated in cell proliferation and invasion[96]. IPMN cells, as also PDA cells, express higher level of S100A6 than normal or pancreatitis-associated epithelial cells[96]. The alteration of S100A6 expression is an early event in pancreatic carcinogenesis; then, its expression gradually increases and so it may be considered a biomarker for the malignant potential. Measuring S100A6 in pancreatic juice may allow to detect early pancreatic cancer or to identify individuals with high-risk pancreatic lesions[96].

Protein S100 calcium binding protein A11 (S100A11),

encoded by the *S100A11* gene, plays an important role in the DNA damage response through a nucleolin-mediated translocation from the cytoplasm into the nucleus that correlates with an increased cellular p21 (the cell cycle regulator) protein level[97]. The levels of S100A11 in bulk tissues and in pancreatic juice are higher in PDA and in IPMN than in non-neoplastic samples, while, in microdissection analysis, IPMN cells are detected to express higher levels of S100A11 than PDA cells do[98]. This behavior suggests that S100A11 could be a tumor suppressor gene and its expression may decrease during progression from a benign to a malignant phenotype. The measurement of S100A11 expression in pancreatic juice may be useful for an early detection of pancreatic cancer, in particular for patients with high-risk lesions or chronic pancreatitis and patients with a family history of pancreatic cancer[98].

Correlation between mutation of the various genes and the different grades of dysplasia of intraductal papillary mucinous neoplasms is shown in Table 3.

ABERRANT EXPRESSION OF MICRORNAS

MicroRNAs (miRNAs) are small (18-24 nucleotides), single-stranded RNA molecules that regulate gene expression by binding messenger RNAs of genes at the 3' untranslated region, resulting in degradation of the target messenger RNA or inhibition of translation[99]; so, the principal function of miRNAs is to regulate stability and translation of nuclear mRNA transcripts[100]. Their role in the control of proliferation, differentiation and apoptosis and their aberrant expression, leading to promotion of expression of proto-oncogenes or inhibition of tumor suppressor genes in many tumors, implies that they might function as tumor suppressor genes and as oncogenes[99,101-103].

Misexpression of miRNAs is commonly observed in pancreatic adenocarcinoma and its precursor lesions[99,104,105] and in IPMN[99].

The expression of the primary transcripts for miR-21 starts from chromosome 17q23.2; upregulation of miR-21 in cancer cells leads to apoptosis inhibition and acquisition of invasive properties[106]. Between the genes repressed by miR-21, there are the tumor suppressor phosphatase and

tensin homolog (PTEN), with the consequential activation of the Akt signaling pathway[107], and the *PDC4*, with the resulting promotion of cellular transformation and metastases[108].

miRNA-155 instead is co-expressed in conjunction with the non-coding transcript BIC, from chromosome 21q21.3[109]. Misexpression of miR-155 in pancreatic cancer represses the function of tumor protein 53 induced nuclear protein 1 (TP53INP1), that is pro-apoptotic[110].

miR-21 and miR-155 are significantly upregulated in the majority of non-invasive IPMNs and their expression correlates with histological features of progression[100].

miR-21 and miR-155 appear more up-regulated in invasive IPMNs compared with non-invasive IPMNs and with normal tissues[101], with a significantly greater proportion of IPMNs with carcinoma *in situ* expressing up-regulated miRNA compared to IPMN adenomas[100]. The percentage of miR-155 expression is 83% in IPMNs and 7% in normal ducts, whereas miR-21 expression reaches 81% in IPMNs and 2% in normal ducts[100]. Specifically, all the carcinoma *in situ*-IPMNs expressed miR-155, while it is expressed in 54% of IPMN adenomas, and 95% of IPMNs with carcinoma *in situ* expressed miR-21 while 54% of IPMN adenomas do it[100]. A significant higher proportion of carcinoma *in situ*-IPMNs expressed both miRNAs, compared to adenomas[100].

The association of the high levels of miR-21 with worse overall survival and a shorter median disease-free survival defines miR-21 as an independently prognostic factor for mortality and disease progression[101].

Differently from miR-155 and miR-21, expression of miR-101 is significantly higher in non-invasive IPMNs and normal tissues than in invasive IPMNs[101,111]. miR-101 could downregulate EZH2 (enhancer of zeste homolog-2, expression of which is significantly high in malignant IPMNs) in benign IPMNs, while a reduced level of miR-101 is attributable to increased expression of EZH2 in malignant IPMNs. Hence, low levels of miR-101 could be a trigger for the adenocarcinoma sequence of IPMN by upregulation of EZH2[111].

HUMAN MUCIN GENES EXPRESSION

MUCs are a group of genes that transcribe for high molecular weight glycoproteins (mucins)[112]. Their function is the lubrication and the protection of epithelial mucosa, but they also play a role in homeostasis and carcinogenesis[113,114]. In the last years, core proteins for various human mucins have been identified (MUC1-9, MUC11-13, MUC15-20)[115-117]. Mucins are differentially expressed by several epithelial cells: goblet cells of the intestinal mucosa express overall MUC2 and then MUC3 and MUC4, that are expressed also by enterocytes. As regarding gastric mucosa, surface cells express MUC5AC and pyloric glands MUC6[118,119]. Ductal cells of the pancreas are principally characterized by the expression of MUC3, MUC5B and MUC6[120,121]. In pancreatic cancer, MUC1 and MUC2 are the more described and are re-

spectively reported as markers of "aggressive" and "indolent" phenotypes[122,123].

MUC1 have an inhibitory role in cell-cell and cell-stroma interaction, mostly through integrins, and plays also a role in immunoresistance of neoplastic cells to cytotoxic T cells[124]. The MUC1 mucin mRNA is detected in several epithelial tissues and it is overexpressed in pancreas[125], where it is commonly expressed in PanIN-3 (61%)[126] and in ductal adenocarcinoma. Only rarely MUC1 is detectable in non-invasive, non-pancreatobiliary-type IPMN and colloid carcinoma[127]. In IPMNs, MUC1 expression has a specificity of 90% for the presence of tubular type invasion[126]. The expression of MUC1 in IPMNs is low, mainly in lower grade lesions; indeed, MUC1 expression is higher as the degree of dysplasia improves[126]. MUC1 expression is detectable in 8.6% of adenoma/borderline IPMNs and in 35.8% of carcinoma IPMNs[128]. High levels of MUC1 expression, its aberrant intracellular localization and changes in glycosylation are associated with an aggressive phenotype[127]. MUC1 overexpression is considered the most sensitive and specific marker of invasive carcinoma[129]. It is infrequent to detect MUC1 in cystic fluid of IPMNs[130].

MUC2 is a secretory mucin, associated with gel formation *via* polymers that are linked end to end by disulfide bonds and regulates cell proliferation *via* cysteine-rich domains[126]. It is expressed in the perinuclear region of the goblet cells of normal mucosa of the colon, small intestine and airways[112,131,132], providing an insoluble mucous barrier and protecting epithelium. It is never expressed in normal pancreatic tissue, but it is detectable in intestinal-type IPMN and colloid carcinoma[23,130]. MUC2 in cystic fluid is a marker of the intestinal subtype and delineates high-grade dysplasia/invasive cancer[127,133].

The expression of MUC2 rises from early IPMN (adenoma and borderline) to higher grade IPMN (carcinoma *in situ*) to colloid carcinoma (30%, 54% and 100%, respectively)[126], in contrast to ordinary ductal adenocarcinomas of the pancreas, where 63% are MUC1+ and only 1% is MUC2+[123,126,134].

In case of invasive carcinomas developing from IPMN, colloid carcinomas are exclusively MUC2+ (100%) and MUC1-, whereas 60% of tubular adenocarcinomas express MUC1 and only 1% of them expresses MUC2. In the IPMNs with MUC2+ expression, a carcinomatous component has been observed in the 89% of the cases, whereas, in case of MUC2- expression, it has been observed in only the 19% of the cases. Invasive carcinoma is more frequent in the IPMNs with MUC2+ expression (67%) than in the IPMNs with MUC2- expression (6%), so the clinical outcome for patients with IPMN with MUC2+ pattern tended to be worse than for those with IPMN with MUC2- pattern[112].

IPMNs with MUC2- pattern have low incidence of carcinoma in tumors under 4 cm in diameter, whereas IPMNs with MUC2+ pattern have a high incidence of carcinoma even in small tumors. Hence, the incidence of carcinoma in the MUC2- tumors is correlated with

Table 4 Mucin expression profiles of the histological subtypes of intraductal papillary mucinous neoplasm

	MUC1	MUC2	MUC5AC	MUC6
Gastric	-	-	+	-
Intestinal	-	+	+	-
Pancreatobiliary	+	-	+	+
Oncocytic	-	-	+	+

MUC: Mucin.

tumor size, while in the MUC2$^+$ tumors there is no correlation with tumor size. In the light of this behavior, it is unlikely that MUC2$^+$ tumors are derived from MUC2$^-$ tumors; so, the two types of tumors arise from different cell lineages[112].

MUC4 is a transmembrane ligand for ErbB-2, functioning in cell-cell and cell-extracellular matrix interactions[135]. Through alteration of cell properties and modulation of ErbB-2 expression, it acts on tumor growth and metastases[136]. MUC4 is not detectable in normal pancreatic tissue and it is expressed more frequently in high-grade and invasive IPMNs, with a worse overall survival in pancreatic cancer[136,137]. MUC4 is secreted into the cyst fluid and, in high-risk cysts, it is present in higher concentrations[127,136]. This increase in high-risk cysts could be reflective of activation of the ErbB-2 signaling pathway that may transform borderline cysts to a malignant phenotype[127,136]. Hence, MUC4 could be involved in the progression of adenoma cells to more aggressive cells and to be considered a diagnostic molecular marker indicating the existence of cellular atypia[136].

MUC5AC is a secretory mucin abundantly present in the stomach on the gastric mucosa[138]. Its aberrant expression is present in Barret esophagus[139], in gastric metaplasia in the duodenum[140] and in the colonic adenoma or cancer[131,138]. It seems to form a protective gel around tumors[141], that may be advantageous for all neoplasms. As MUC2, MUC5AC is never expressed in the normal pancreatic tissue, but is detectable in all types of IPMN and in PanINs[127,136]. The increase of expression of MUC5AC is an early event in pancreatic carcinogenesis[142]. MUC5AC is involved in the transition of hyperplastic cells to adenoma cells, so it could be a unique marker to differentiate IPMN tumor lesions from hyperplastic lesions, for its presence in all IPMN lesions, irrespective of histologic grades and epithelial subtypes[128,136].

The synchronous expression of MUC4 and MUC5AC in about half of adenoma IPMNs and almost all borderline and malignant IPMNs suggests that they act cooperatively to the carcinogenesis of IPMNs[136].

MUC6, a pyloric-type mucin, plays an important role in foregut differentiation of cells and it is implicated in gastric and pancreatic carcinogenesis[142]. Its expression is detectable in the intercalated ducts of the pancreas, in the small pancreatic tributary ducts with pyloric features and in Brunner glands of the duodenum, with focal granular labeling in some acini[134,143].

MUC6 seems to be regulated by key molecules such as Sp and NFκB, known for their important roles in pancreatic tumorigenesis[144,145].

The direction of differentiation in the various histological types of IPMNs is reflected in the expression of mucins: MUC1 is expressed in pancreatobiliary-type, MUC2 in intestinal-type, while MUC5AC is typically expressed in gastric-type IPMN. MUC5A can also be found, in combination with MUC1, in pancreatobiliary and oncocytic types or, in combination with MUC2, in the intestinal type IPMN[18,146]. As regarding MUC6, IPMNs characterized by oncocytic-type papillae show diffuse and strong MUC6 expression; IPMNs with pancreatobiliary-type papillae also consistently express MUC6, but the degree of expression is less intense than in oncocytic samples[143].

The feasibility of evaluating MUC expression from material obtained by endoscopic ultrasound-guided fine-needle aspiration has been assessed[147,148]; RNA can be extracted from biopsies obtained under endoscopic ultrasound with fine needle aspiration (EUS-FNA). A relationship between the abnormal expression of some classes of MUCs and IPMNs has been demonstrated[147,148]; MUC7 could be a potential biological marker to identify malignant lesions[147] (Table 4).

DISCUSSION

The data exposed so far must be applied to the clinical management of IPMNs in order to differentiate low-risk from high-risk lesions and to determinate which patients need surgery and which can be maintained under surveillance.

EUS-FNA of pancreatic cyst fluid sampling could be the procedure that enables to discriminate between benign and malignant IPMNs. The information we can obtain from cystic fluid analysis is various. Some authors proposed to measure the levels of carcinoembryonic antigen (CEA) concentration, but actually CEA is suitable only for the diagnosis of a mucinous cyst and not for the distinction of the degree of dysplasia[149,150].

More information instead can result from DNA analysis of pancreatic cystic fluid: DNA quantification, allelic imbalance/loss of heterozygosity and point mutations. The most significant markers in differentiating malignant IPMNs from benign IPMN lesions, among the genes presented in this paper, are MUC2, CDKN2A/p16, hTERT, S100A4 and Shh. In light of the percentage values of mutation in relation to the degree of dysplasia found in Literature, the analysis of DNA mutation detailed for these genes could be helpful to delineate IPMNs with high-grade dysplasia or invasive cancer that need surgical resection and adenoma and borderline IPMNs that can be maintained in follow-up.

In literature, KRAS is frequently reported as a possible indicator of malignancy in IPMNs; however, the lack of significant difference among the incidence of KRAS mutation in the various degrees of dysplasia makes KRAS inadequate in identifying benign and malignant IPMNs, being highly specific only for mucinous
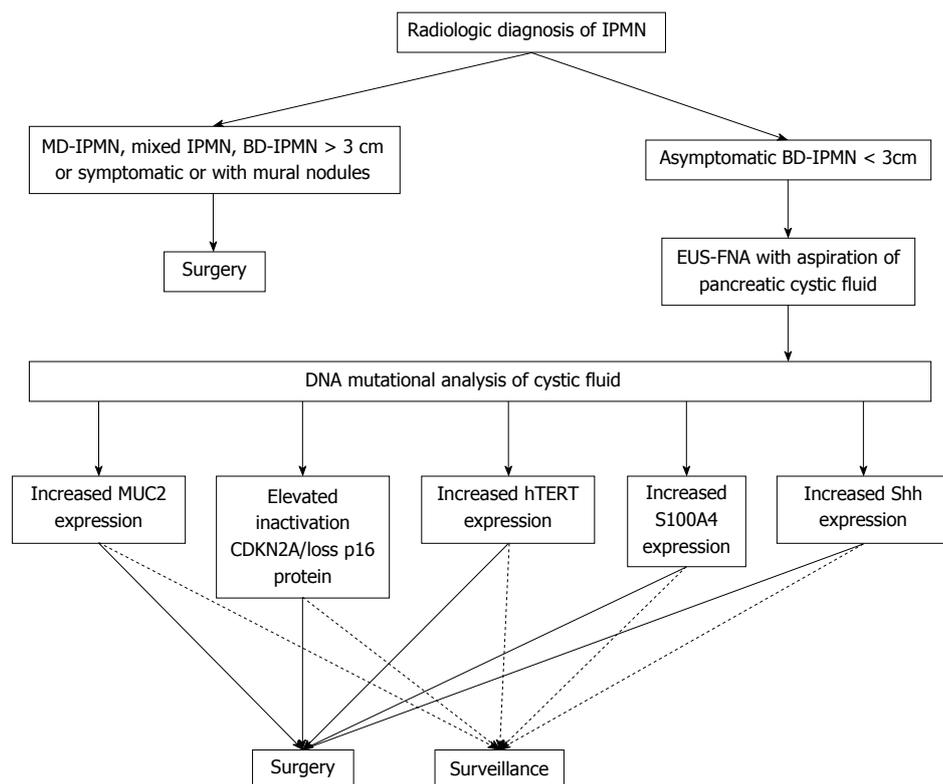
Figure 1 Algorithm for the management of patients with intraductal papillary mucinous neoplasm created on the base of molecular genetics data presented in the paper (the continuous line stands for "yes", the dashed line stands for "no"). IPMN: Intraductal papillary mucinous neoplasm; EUS-FNA: Endoscopic ultrasound with fine needle aspiration; hTERT: Human telomerase reverse transcriptase.

differentiation of pancreatic cysts[151] (Figure 1).

CONCLUSION

As stated above in this paper, the knowledge of molecular biology of IPMNs has impressively developed over the last few years, but more research is needed to use this information for clinical intent, in order to better define the natural history of these diseases. In addition, IPMN represent a very interesting histological and molecular model of pancreatic neoplasm, and, as it may be considered as precursor of PDA, new insights into the molecular pathology of this neoplasm could be of interest for the understanding of the biology of PDA.

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2014 ADVANCES IN PANCREATIC CANCER

Pancreatic cancer-improved care achievable

Trond A Buanes

Trond A Buanes, Division of Cancer, Surgery and Transplantation, Institute of Clinical Medicine, Faculty of Medicine, Oslo University Hospital (OuS), University of Oslo, 0316 Oslo, Norway

Trond A Buanes, Department of Gastroenterological Surgery, Division of Cancer, Surgery and Transplantation, Oslo University Hospital, 0316 Oslo, Norway

Author contributions: Buanes TA contributed to the manuscript.

Correspondence to: Trond A Buanes, MD, PhD, Professor of Surgery, Division of Cancer, Surgery and Transplantation, Institute of Clinical Medicine, Faculty of Medicine, Oslo University Hospital (OuS), University of Oslo, P.O. Box 1072 Blindern, 0316 Oslo, Norway. trond.buanes@medisin.uio.no

Telephone: +47-230-70958   Fax: +47-911-82938

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Abstract

Pancreatic adenocarcinoma is one of the most aggressive cancers, and the decline in mortality observed in most other cancer diseases, has so far not taken place in pancreatic cancer. Complete tumor resection is a requirement for potential cure, and the reorganization of care in the direction of high patient-volume centers, offering multimodal treatment, has improved survival and Quality of Life. Also the rates and severity grade of complications are improving in high-volume pancreatic centers. One of the major problems worldwide is underutilization of surgery in resectable pancreatic cancer. Suboptimal investigation, follow up and oncological treatment outside specialized centers are additional key problems. New chemotherapeutic regimens like FOLFIRINOX have improved survival in patients with metastatic disease, and different adjuvant treatment options result in well documented survival benefit. Neoadjuvant treatment is highly relevant, but needs further evaluation. Also adjuvant immunotherapy, in the form of vaccination with synthetic K-Ras-peptides, has been shown to produce long term immunological memory in cytotoxic T-cells in long term survivors. Improvement in clinical outcome is already achievable and further progress is expected in the near future for patients treated with curative as well as palliative intention.

Core tip: Curative treatment outcome for patients with pancreatic cancer is achievable if early surgical treatment is combined with adjuvant chemotherapy. Nevertheless, most patients end up in a palliative situation, earlier or later. Also palliative therapeutic interventions are improving, but a multidisciplinary team with advanced expertise is a prerequisite for optimal care.

INTRODUCTION

Pancreatic adenocarcinoma is one of the most aggressive cancers. Despite all advances in cancer treatment it is still the fourth most-frequent tumor-related cause of death in the Western world[1]. The reasons for this are challenges associated with the diagnosis, which tend to be late and precarious, but more importantly limited therapeutic options. Therefore, even though cancer mortality in Europe has declined by approximately 10% during recent years, this is not the case for pancreatic cancer[2]. The development of new and potent treatment options is therefore strongly needed. In recent years there

have been important advances in the organization of care for pancreatic cancer patients, also resulting in more focused studies on preoperative investigation, surgical, oncological and immunological treatment. This review summarizes available evidence, focusing best clinical practice based on the latest translational research.

RESEARCH

Search in PubMed was performed with the key words: Pancreatic cancer, combined with pathogenesis, prevention, diagnosis and treatment. Reports were selected, based on publication date (preferring recent studies) and conceived internal validity in each single paper. A balanced mix of original papers, preferring randomized trials initiated after 2003, and Cochrane reviews, meta-analyses and review articles, relevant to the scope of this review, were prioritized.

PATHOGENESIS

The cause of pancreatic cancer remains unknown. Several environmental factors have been implicated, but a causal role has been shown only for tobacco. The risk of pancreatic cancer in smokers is 2.5 to 3.6 times that in non-smokers, increasing with greater tobacco use and longer duration of exposure[3]. Worldwide the proportion of early onset pancreatic cancer is strongly correlated with lung cancer mortality[4] ($r^2 = 0.53$), suggesting that approximately half of the variation in the proportion of early onset pancreatic cancer can be explained by smoking. The possible roles of moderate intake of alcohol, coffee, and use of aspirin as contributing factors are supported by very limited data. Increased risk of pancreatic cancer among patients with blood type A, B or AB as compared with blood type O has been observed in recent reports[5,6]. Pancreatic cancer also occurs with increased frequency among persons with long-standing diabetes[7,8], but this does not necessarily imply that diabetes is a pathogenetic factor, as it may be a consequence of the cancer. The latter concept is supported by the recent observation that adrenomedullin is upregulated in patients with pancreatic cancer and causes insulin resistance in β cells[9]. A recent meta-analysis also favors the association between hepatitis B/C infection and pancreatic cancer[10].

There may be a causal relationship between chronic pancreatitis and pancreatic cancer, but the population attributable fraction was estimated to only 1.34% (95%CI: 0.612-2.07) in a recent study[11], suggesting that a relatively small proportion of pancreatic cancers might be avoided if pancreatitis could be prevented. Pancreatitis appearing shortly before the diagnosis of pancreatic cancer is probably the result of tumor-related ductal obstruction. But patients with hereditary pancreatitis, which is a rare subgroup of chronic pancreatitis, have a marked relative and absolute increased risk of pancreatic cancer[12] as compared to the general population,

especially in smokers. This has been documented in two comprehensive international studies[13,14]. Whitcomb[15] identified in 1996 the first genetic defect in patients with hereditary pancreatitis on the cationic trysinogen gene (*PRSS1*).

GENETICS

Pancreatic cancer has been shown to result from a successive accumulation of gene mutations[16] in the ductal epithelium, evolving from premalignant lesions to fully invasive cancer. Pancreatic intraepithelial neoplasia is a precursor of pancreatic cancer[17], progressing from minimally dysplastic epithelium to invasive carcinoma. During carcinogenesis accumulation of mutations take place, initially activation of the *KRAS2* oncogene, then inactivation of the tumor suppressor gene CDKN2A and inactivation of the tumor suppressor gene *TP53* and finally deletion of the SMAD family member 4 gene[18,19].

At least one of four genetic defects are present in almost all patients with fully established pancreatic cancer[20]. Activated mutations in the *KRAS2* oncogene is very frequent in pancreatic cancer cells, making this mutation an appropriate target for immunological attack from vaccine-activated cytotoxic T-cells[21]. The abnormal Ras protein, generated from transcription of the mutant *KRAS* gene, results in permanent activation of proliferative and survival signaling pathways in the cancer cells.

Comprehensive genetic analysis of 24 pancreatic cancers showed that the genetic basis of the tumor is extremely complex and heterogeneous[22]. An average of 63 genetic abnormalities per tumor was found, mainly point mutations, classified as likely to be carcinogenetically relevant. These abnormalities can be organized in 12 functional pathways. A model of this carcinogenetic process is presented graphically as the "Components of Pancreatic Cancer" in a clarifying review article by Hidalgo[19].

Genomic sequencing, evaluating the clonal relationships among primary and metastatic pancreatic cancer cells, has recently been performed. Based on differential accumulation of mutations, the authors estimated that pancreatic tumors cells are present for 6 to 12 years before development of metastatic disease, suggesting a broad time window for early detection of the primary tumor[23].

PREVENTION

Universal primary screening for pancreatic cancer is currently not recommended, given the tools available and their performance[24,25], even though the time interval when pancreatic cancer cells are present in advance of their dissemination, is probably long[23,26]. Hence, the beneficial potential of a biomarker panel with the required accuracy, is huge. No imaging modality fills this requirement. Sensitivity as well as specificity of endoscopic ultrasound (EUS) examination has been improving during recent years, and enables fine needle aspira-

tion during the same procedure. But even screening of high-risk groups by EUS combined with computed tomography (CT), should only be performed in the context of prospective trials[25,27].

The number of patients with incidentally diagnosed cystic pancreatic lesions is rising, most likely due to the increased use of high-resolution imaging[28]. The variable degree of malignancy potential in different cystic pancreatic lesions can be clarified by EUS guided aspiration/analysis of cystic fluid, as low levels of carcinoembryonic antigen (CEA) in cyst fluid from serous cystadenoma has been documented[29]. Oppositely, cyst fluid from mucinous lesions tends to have high CEA values[30]. Intraductal papillary mucinous neoplasms (IPMN) in the main duct develop invasive carcinoma more often than IPMN lesions in side branches[31], both supposed to be more indolent than sporadic pancreatic adenocarcinoma. But in patients with lymph node metastasis, long term survival curves are almost identical[32]. IPMN-lesions usually have a premalignant time interval of several years duration. Surgical resection of mucinous cystic lesions before they become invasive carcinoma, apparently represents one of the best opportunities to prevent pancreatic cancer. Also when incidentally recognized malignant lesions undergo surgical resection, survival is significantly improved[33]. Five year survival above 30% is reported after resection of incidentaloma, even in distal pancreatic carcinoma[34]. Prevention of death from pancreatic cancer is therefore increasingly affordable, even though screening programs have not become the way to do it up till now.

DIAGNOSIS

High quality imaging plays a crucial role in the diagnosis of pancreatic tumors. One cross-sectional imaging modality is sufficient for adequate evaluation of tumor diagnosis and resectability in most patients. Multidetector CT angiography, performed by using a dedicated dual-phase pancreatic protocol[35] is the preference of most centers[36]. Adoption of a standardized template for radiology reporting in pancreatic neoplasms is strongly recommended in a consensus statement, authored by radiologists, gastroenterologists and hepatopancreaticobiliary surgeons under the sponsorship of the Society of Abdominal Radiologists and the American Pancreatic Association[35]. Magnetic resonance imaging (MRI), including magnetic resonance cholangiography, may help to differentiate cystic lesions, but does not add information about resectability. EUS guided fine-needle aspiration is essential for analysis of cystic fluid, and is the best method for obtaining a tissue diagnosis when needed, *i.e.*, before neoadjuvant or palliative chemotherapy. Routine use of endoscopic retrograde cholangiopancreatography (ERCP) or ^{18}F-fluorodeoxy-glycose (^{18}F-FDG) PET cannot be recommended[36,37]. ERCP should only be used for therapeutic purposes, because of the high frequency of severe complications. Proce-

dure-related mortality rate of 1.4% have recently been published[38]. Routine preoperative biopsy of resectable pancreatic tumors is not advisable, because malignant disease cannot be ruled out reliably[39]. Seeding of cancer cells along the path of the needle[40] after percutaneous biopsy is another reason for avoiding preoperative biopsy in patients with resectable tumors.

BIOMARKERS

Numerous biomarkers for cancer have been developed[41], but the clinical benefit in pancreatic cancer patients has so far been limited, and the persistent search for a biomarker panel with improved sensitivity/specificity is important. New markers based on analysis of gene expression[42], proteomic analysis[43], radiolabeling with anti-Claudine 4[44] and membrane bound molecules[41] are developing, but a panel also including microRNA (miRNA) as a biomarker, seems presently most likely to obtain clinical significance[45]. The beneficial role of a secure biomarker panel is obvious for primary diagnosis, as well as monitoring of treatment outcome. Carbohydrate antigen 19-9 (CA 19-9) is in widespread clinical use, even though sensitivity and specificity are low[46]. Its clinical usefulness in early detection of recurrent disease and therapeutic monitoring is well documented[47].

STAGING

Pancreatic cancer is staged according to the most recent edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) classification[48]. Treatment of different stages have been changing during recent years, and the outcome, recorded with survival and Quality of life (QoL) as clinical endpoints, changes with the development of revised guidelines. Bilimoria *et al*[49] reported in 2007 survival data resulting from treatment according to staging by the 6th edition of AJCC Pancreatic Cancer Staging System, when T1, T2 and T3 tumors are considered potentially resectable, even though locally advanced T3 tumors involve the superior mesenteric veins (SMV), portal vein (PV), or splenic vein (SV). Median survival 24.1 mo was reported in stage 1A, decreasing to 4.5 mo in stage IV. Details of stage characteristics are given in Bilimoria *et al*[49]'s report from the National Cancer (NCD)-database and in Hidalgos *et al*[19]'s review. However, the practical consequences of staging, related to assessment of resectability and timing of an operation, is changing after the introduction of the concept of borderline resectable tumors. As indicated in Table 1, this question depends on the local handling program of each pancreatic center.

TREATMENT

Long term survival is not achieved in pancreatic cancer patients without surgical resection of the tumor. This is true even when chemoradiation is used in early stage

Stage	Tumor grade[1]	Nodal status[1]	Distant metastases[1]	Resectability
I A	T1	N0	M0	Resectable
I B	T2	N0	M0	
II A	T3	N0	M0	Borderline resectable[2]
II B	T1, T2 or T3	N1	M0	
III	T4	Any N	M0	
IV	Any T	Any N	M1	Unresectable, independent of T-grade

[1]N denotes regional lymph nodes, M distant metastases, and T primary tumor; [2]The concept "borderline resectable", related to T3 and T4 tumors, is not uniformly conceived between pancreatic centers.

disease[50,51]. The superiority of surgery over chemoradiotherapy has even been documented in a randomized controlled trial (one year survival 62% vs 32%, $P < 0.05$)[52]. In the Surveillance Epidemiology and End Result (SEER) database, curative intent surgery was found to be the strongest predictor of prolonged survival[53]. In high-volume centers, resection of mesenteric vessels and even multivisceral resection are performed in patients with locally advanced disease[54-58] to enable long term survival.

MULTIDISCIPLINARY APPROACH

A multidisciplinary approach is mandatory, already at the time of primary diagnosis of pancreatic cancer. The impact was evaluated in 203 consecutive patients at the Johns Hopkins pancreatic multidisciplinary clinic in 2006/2007[59], and a comprehensive and coordinated evaluation led to changes in therapeutic recommendations in almost one-quarter of patients. Patient logistics and care was organized around a nurse navigator in an "All-in-One Resort"[60,61], and high degree of patient satisfaction was reported. The continuous ongoing development of oncological and surgical treatment algorithms in patients with borderline resectable and locally advanced disease, further underlines the importance of close cooperation within the multidisciplinary team in order to maximize short- and long-term oncological outcomes[62].

SURGERY - FOR WHOM, HOW AND WHEN?

Improved quality of preoperative staging has enabled radiological classification of pancreatic tumors as resectable, borderline resectable (a concept which has been defined by a consensus panel[63,64]) and locally advanced unresectable tumors. Borderline resectable tumors may be treated by neoadjuvant chemoradiation, which has been shown to result in high rates of R0 resections, and

5 years survival in the same range as primary resectable tumors[63]. But the downside of the neoadjuvant protocol was that significant numbers of included patients with potentially resectable tumors at inclusion (approximately ¼), progressed during neoadjuvant treatment and could never be resected[63,65]. These patients have the disadvantage of median survival 8 mo[66]. Future development of care for patients with pancreatic cancer is obviously emerging towards more advanced surgery for new patient groups combined with oncological efforts after surgery, probably also preoperatively. Further prospective clinical studies, focusing clinical outcome, are mandatory. But the underutilization of surgery in patients with localized pancreatic cancer is a major ethical problem: Numerous patients without any contraindication against surgery never receive surgical treatment of their serious disease[50,67].

The best outcome of surgical treatment is histologically free resection margin (R0) and it has been unclear whether an R1 resection confers any survival benefit at all over no surgical removal of locally advanced tumors. Even the predictive value of an R0 resection has been queried: In 360 consecutive patients, undergoing pancreaticoduodenectomy, R0 was found in 300 (83.3%), but R0 status did not come out as survival predictor in multivariate analyses[68]. Patients who underwent an R1 resection had a median overall survival of 21.5 mo compared with 27.8 mo after R0 resection, which was found not significantly different. This might in part be explained by the fact that up till 2006, pathological examination of pancreaticoduodenectomy specimens were not standardized between different pancreatic centers[69,70]. Verbeke et al[71] published a systematic, detailed technique for handling and evaluation of resected specimens with colouring of the resectional margins, redefining R1 resection as tumor cells within 1 mm of the resection margin. The Heidelberg group documented that R0 resection came out as predictor of long term survival in multivariate analyses after the introduction of this standardized handling of resected specimens[54]. Accordingly, refinement of surgical technique, aiming at increased rates of R0 resection, defined by new standards, is mandatory. This may require increased rates of resection of the SMV/PV[72], altered dissection strategy[73] or it might be advantageous to alter the whole treatment algorithm, introducing neoadjuvant chemoradiotherapy in patients with borderline resectable tumours, as described in the United States National Comprehensive Cancer Network guidelines[36,74]. European guidelines are different, as neoadjuvant chemoradiotherapy is not recommended in patients with resectable pancreatic cancer[75]. The intention behind the neoadjuvant treatment algorithm is to avoid surgery in patients with rapidly progressive disease, and to achieve better local tumour control for the residual group, potentially even to down-size unresectable locally advanced tumours to allow secondary resection. Chemoradiotherapy before any surgical resection selects patients with more stable disease for surgery and putative

Table 2 Core data, characterizing outcome of neoadjuvant chemoradiation and surgery, *vs* upfront surgery plus adjuvant chemotherapy in patients with resectable and/or borderline resectable pancreatic tumor

Ref.	Patients included	Inclusion periode	Treatment algorithm	Proportion not resected	R0 status	Frequency of vascular resection	Median survival in months
Katz *et al*[63] 2008	160	1999-2006	Neo-adjuvant	59%	94%	27%	40
Nordby *et al*[84] 2013	135	2008-2010	Upfront surgery+ advance	11%	42%	6%	Na1
Neop-tolemos *et al*[76] 2010	1088	2000-2007	Upfront surgery+ advance	Only resected patients included	65%	17%	23
Schmidt *et al*[77] 2012	132	2004-2007	Upfront surgery+ advance	Only resected patients included	61%	Na2	28

Na1: Too short follow up; Na2: Not given in the paper.

micrometastasis may be treated at an earlier stage. On the other hand significant numbers of primary resectable patients become unresectable during neoadjuvant treatment and the outcome of primary resection followed by adjuvant chemotherapy is lost in many of these cases. A median survival of 23 mo[76] to 28 mo[77] has been documented in two recent randomized controlled trials. This life expectancy is replaced by the prospects of an unresectable tumor, being less than a year, ie. significantly shorter[78].

COMPARISON OF OUTCOME

Table 2 puts together core data from four studies illustrating principal difficulties, arising when outcome of neoadjuvant treatment is compared with upfront surgery followed by adjuvant chemotherapy. Katz *et al*[63] published 2008 median postoperative survival 40 mo and 94% R0 resections, which is the best outcome for resected patients. But most patients included in the study could never be respected, and it is an open question what the clinical outcome of an earlier operation would have been in these cases. On the other hand, Nordby *et al*[72] published in 2013, that almost 90% of patients scheduled for upfront surgery were actually resected, but the rate of R0 resection was low, and it is underlined that alteration of surgical technique might be an opportunity of improvement: Increased frequency of resection of the PV/SMV and/or artery first dissection strategy. Finally, ESPAC centers resected PV/SMV in 17 % of operated patients, and obtained similar oncological outcome in the whole group of included patients, when surgery was performed first. Also the Heidelberg group (Schmidt 2012) has reported equivalent survival after upfront surgery. The different outcome in these series is probably explained by diverse patient selection, differences in preoperative and intraoperative criteria for resectability and variable surgical technique. These parameters illustrate important confounding factors when outcome is compared between neoadjuvant and upfront surgical treatment algorithms. Further efforts are therefore needed to standardize and clarify critical determining factors of outcome in advance of future randomized clinical trials. According to the current available evidence, neoadjuvant therapy is usually not recommended for patients with curatively re-

sectable pancreatic cancer[36,75], but the prospective evaluation in well-designed controlled trials is mandatory. A synthesis of the considerations above is summarized in Figure 1A, suggesting upfront surgery for all patients with resectable tumor, followed by adjuvant chemotherapy. However, the inclusion of resectable as well as borderline resectable tumors in a neoadjuvant protocol is the preference of MD Anderson Cancer Center[79] as shown in Figure 1B. Further details on tumor biology, enabling personalized medical treatment plans would significantly improve outcome in both arms of these trials, and probably reduce health care costs[80,81].

The purpose of surgical resection of pancreatic tumors is radicality, but final R1 status occurs in all centers. Already in 1996 Lillemoe published data, suggesting a survival benefit of R1 resection over locally advanced unresectable tumours[82]. Two recent publications have verified increased survival after R1 resection. Konstantinidis *et al*[83] found median survival 14 mo in 157 R1 resected patients *vs* 11 mo in 286 locally advanced, unresectable cases. Nordby *et al*[84] found median 18 mo survival after R1 resection *vs* 8.1 mo in the locally advanced unresectable group and also QoL, recorded longitudinally, was found improved in the resected group. Collective evidence supports the concept that there is a significant clinical benefit of removing the pancreatic tumor, even if the resectional status is R1.

ONCOLOGICAL TREATMENT

Pancreatic adenocarcinoma tend to be chemoresistant and for a long period little progress has been obtained by traditional anti-tumor treatment, illustrated by the fact that gemcitabine has been standard of care since 1997[85]. But Conroy *et al*[86] published 2011 a randomized controlled trial including 342 metastatic patients, comparing FOLFIRINOX (oxaliplatin, irinotecan, leucoverin and fluouracil) with gemcitabine, and found significantly increased survival (11.1 mo *vs* 6.8 mo). The objective response rate was 31.6% in the FOLFIRINOX group *vs* 9.4% in the gemcitabine group ($P < 0.001$). During ASCO 2012 the FOLFIRINOX regimen was characterized as a paradigm shift in oncological treatment of pancreatic cancer, and this regimen is now under evaluation in potentially curative subgroups, as those with
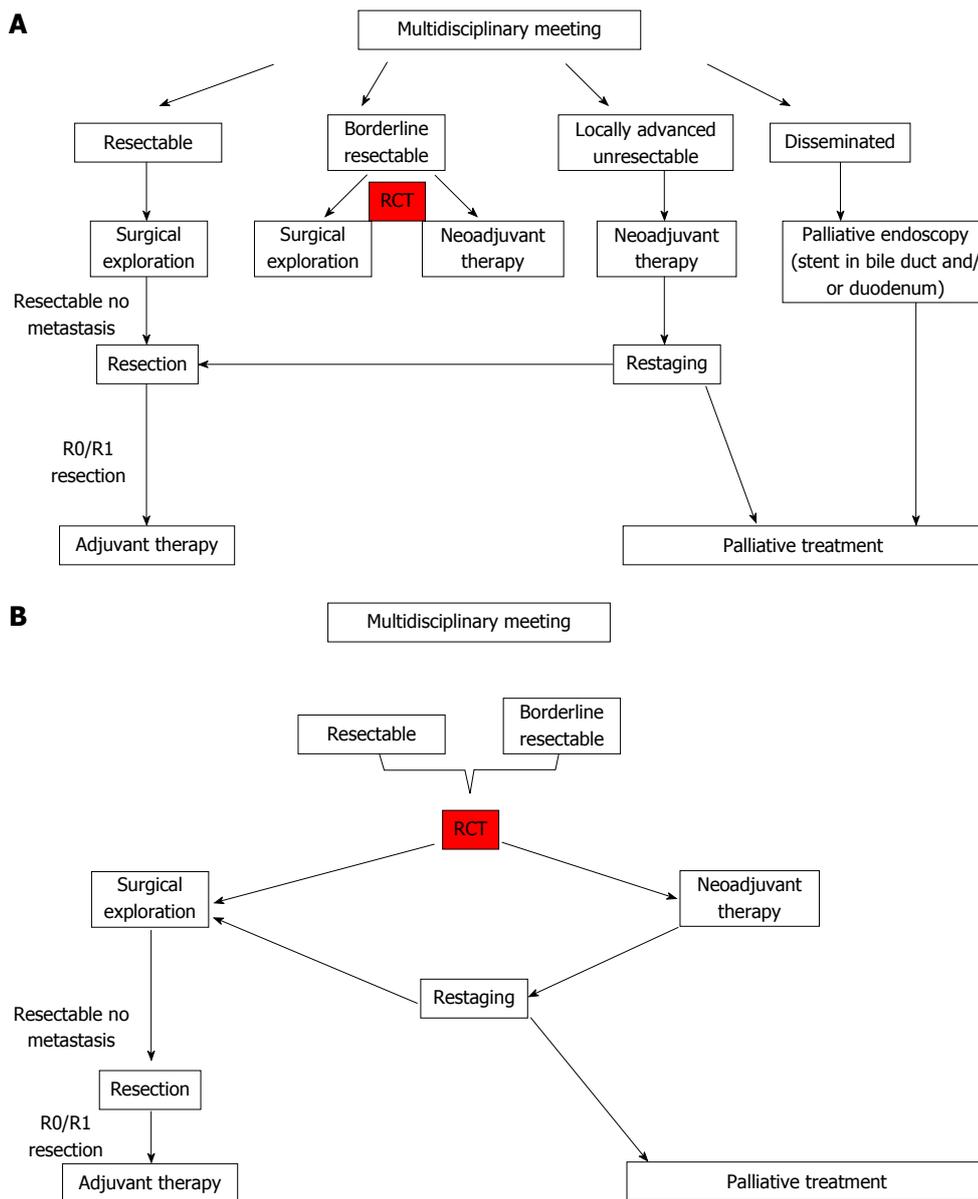
Figure 1 Treatment algorithm for pancreatic tumors including only patients with borderline resectable lesions in randomized controlled trial (A), alternative model, including primary resectable and borderline resectable tumors in randomized controlled trial, comparing outcome of upfront surgery and neoadjuvant chemotherapy (B).

borderline resectable tumours[87]. The results are positive, but the toxicity of FOLFIRINOX generates significant limitations, as only patients with relatively good health can be included.

Evaluation of adjuvant chemotherapy, combined with radiotherapy or alone, was first analysed in well-designed randomized trials in the ESPAC 1 study which showed no survival benefit for adjuvant chemoradiotherapy but a significant prolonged survival in patients treated by fluouracil and folic acid[88,89]. However, a meta-analysis suggests that further studies with chemoradiation is warranted in patients with positive resection margins, as chemotherapy appears relatively ineffective in this sub-group[90]. Another important observation in the ESPAC 1 study was that also QoL, recorded prospectively, was not negatively affected by adjuvant chemotherapy compared to surgery alone[91]. Also adjuvant gemcitabine was found to delay recurrence after complete resection of pancreatic cancer[92]. Finally adjuvant gemcitabine was compared with fluouracil/folic acid in the ESPAC 3 trial, which did not find any difference[76].

Neoadjuvant chemoradiotherapy seems to have an obvious place in patients with locally advanced unresectable tumors, who may become resectable after downstaging[93]. However, downstaging filling the RECIST (Response Evaluation Criteria in Solid Tumors) criteria, was found very rare in a recent review[79]. In patients with resectable or borderline resectable tumors, the role of gemcitabine is controversial[94] due to objective response rates below 10%. But potent new regimens like FOL-

FIRINOX may expand this window of opportunity.

Immunotherapy

After decades of disappointment, the recent success of immunotherapy in metastatic melanoma, including proof-of concept trials[95], have renewed the interest in this form of therapy also against pancreatic cancer. In fact, the concept of immune attach against pancreatic tumor cells by CD4+/CD8+ T-lymphocytes was published already in 1997[96]. A second treatment protocol was initiated simultaneously, utilizing adjuvant vaccination with synthetic ras peptides encompassing residues 5-21 of p21 ras in patients operated for pancreatic cancer[21]. This phase I / II trial included 23 resected patients, receiving adjuvant vaccination, subsequently followed till death or for more than ten years. Three patients mounted a memory response immunologically up to nine years after vaccination. Recurrence was found in a fourth patient six years after the Whipple procedure, and her T-cells had then lost their reactivity. After baseline vaccination (1998), she mounted a strong immune response. The evaluation of K-ras peptides in phase II / III trials are ongoing, so far in the Targovax-study, but numerous other basic and translational efforts are in progress[97].

Also the catalytic subunit of telomerase, hTERT, expressed in 85%-90% of human cancer tissue[98], is an attractive "universal" tumour antigen. A synthetic peptide, GV1001, has been tested in unresectable pancreatic cancer patients, with promising outcome: Vaccination initiated CD4+/CD8+ immune response[99] *via* multiple MHC class II alleles. The intermediate dose of GV1001 resulted in immune response in 3/4 of included patients, with significantly increased survival (median 7.2 mo *vs* 2.9 mo) in responding patients. This resulted in a following phase III trial, the Primovax Study, evaluating GV1001 as monotherapy in one arm, compared with standard gemcitabine in the other arm. The intention was to randomize 520 patients to each arm. But the study was closed after inclusion of 360 patients when preliminary data on the deaths of 174 patients showed no survival benefit in the GV1001 group[100]. Another randomized trial with three arms, comparing survival in metastatic pancreatic cancer after gemcitabine plus capecitabine, *vs* gemcitabine plus capecitabine followed by GV 1001 in the second arm and concurrent gemcitabine/capecitabine in the third arm, could neither improve outcome by adding the vaccine[101].

PALLIATIVE SURGERY/ENDOSCOPY

The majority of patients with pancreatic cancer are not resectable at the time of presentation, with life expectancy less than one year for approximately 80%-90%. Palliative interventions for these patients intend to solve problems associated with biliary occlusion and/or duodenal obstruction. The advantage of surgical palliation with double bypass has been to obtain lifelong palliation with one single procedure[102]. But improved radiological staging enables secure prediction of resectability in most

cases, and the advantage of avoiding surgical exploration of unresectable patients favors endoscopic stenting, also of patients with duodenal obstruction[103]. The development of defined quality indicators for the different aspects of the handling of pancreatic cancer patients[104] enables better focus on clinical outcome in future treatment guidelines. The symptom profile of advanced pancreatic cancer is dominated by fatigue and pain[105] and appropriate treatment of nausea and vomiting is important[106]. The palliative functions of the multidisciplinary team have to be closely integrated to offer well-timed help when treatment aspirations change from curative to palliative ambitions[107]: Endoscopic and radiological interventions, together with nutritional support may significantly improve clinical outcome[108].

QUALITY OF LIFE/PATIENT REPORTED OUTCOME

The short survival in most patients with pancreatic cancer makes clinical research difficult due to limited follow-up before transition into a general palliative stage. The symptom profile adds to this problem, because fatigue is a major problem for the majority of patients already at the time of primary diagnosis[105], and several patients are unable to fill comprehensive report forms. Available knowledge about health-related QoL in pancreatic cancer patients is constrained - for these and several other reasons. The lack of disease-specific tools for QoL-registration in patients with pancreatic cancer is one of the main reasons for shortage of information about clinical outcome. Several self-reported measures have been used in research, but only the European Organisation for Research and Treatment in Cancer (EORTC) has developed a disease-specific instrument for pancreatic cancer[109]. The QoL module for pancreatic cancer (EORTC QLQ-PANC26) has 26 questions and must be used in conjunction with the generic instrument EORTC Quality of Life Questioinnaire-C30 (EORTC C-30). Ultimately, altogether 56 questions have to be completed, strongly restricting the feasibility of the instrument both in research and clinical practice. This applies particularly for patients with severe, disabling disease[105]. The Edmonton Symptom System (ESAS) form is short and hence feasible, but generic. A recent new instrument is now developed, which is short and disease-specific, the pancreatic cancer disease impact (PACADI) score[110]. The methodology behind the PACADI score utilized experience from rheumatology, where the Rheumatoid Arthritis Impact of Disease (RAID) score, based on patients' selection of dimensions where the disease has the most important impact on their QoL, has been developed and validated[111,112]. The RAID score is proven to be feasible and is now widely used in research. Similarly, the PACADI score asked for the patients' priorities. The three dimensions with most severe negative impact on pancreatic cancer patients QoL, was pain/discomfort, fatigue and problems with bowel/digestion. But patients
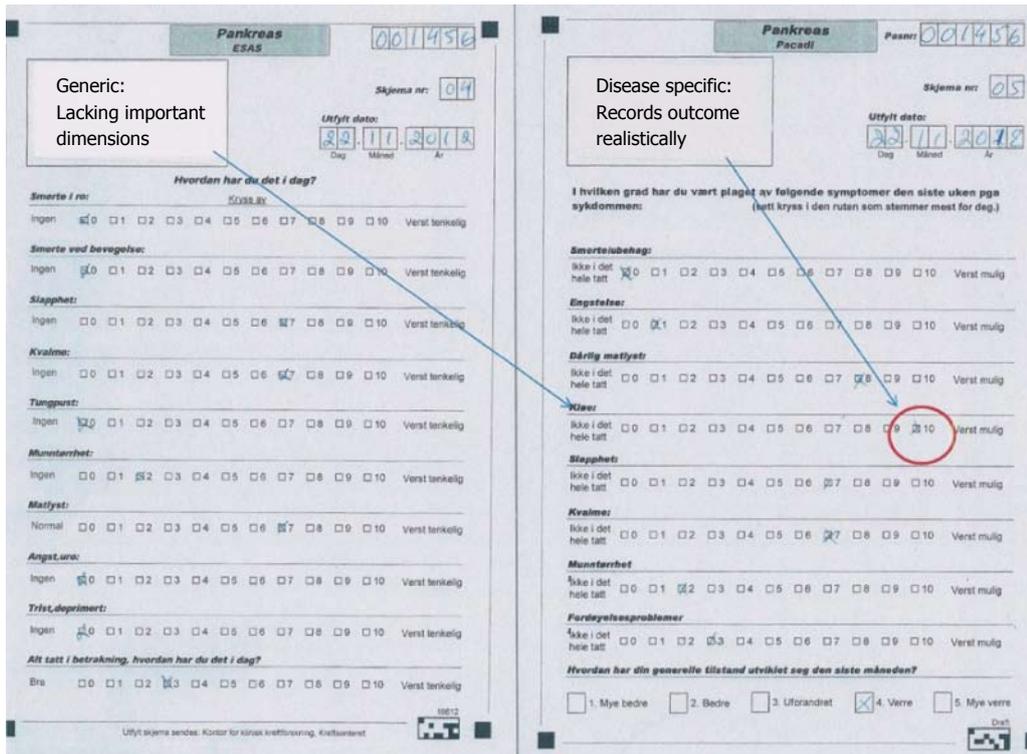
Figure 2 ESAS and pancreatic cancer disease impact form in a patient with serum bilirubin 550 μmol/L, illustrating that only the disease specific instrument enables report on the patient's most severe problem.

with severe icterus reported itching as their most important problem. In order to characterize clinical outcome of therapeutic interventions in a cohort with the short life expectancy of pancreatic cancer patients, it is of utmost importance to obtain valid data on patient reported outcome (PRO). Figure 2 illustrates the difference between a generic (ESAS) and disease-specific (PACADI) instrument in this regard.

IMPORTANCE OF PATIENT VOLUME

Pancreatic surgery has now been accepted as one of the most recognized high-risk, low-volume surgical procedures, but this has not taken place without widespread reluctance in the medical community. One early comprehensive analysis of the relationship between a hospitals patient volume and outcome, was published in 2002 by Birkmeyer et al[113], focused on selected cardiovascular and cancer procedures. Absolute differences in adjusted postoperative mortality rates after pancreatic resections ranged from 16.3% (low volume) and 3.8% (high volume). Several subsequent reports supported the concept that outcome is best in high-volume hospitals, first because complications are recognized earlier and handled better, second because better oncological surgery and chemotherapy is offered[114-116]. The statement that postoperative mortality rates as well as long-term survival are improved with high patient volume, is now clearly evidence-based[117]. The aggressiveness of the tumor combined with the rates and severity grade of complications

associated with pancreatic surgery, resulted in an almost nihilistic therapeutic attitude for several years[118]. The fact that most patients with pancreatic cancer die shortly after diagnosis was for years a "self-verifying prophecy", uphold by negative expectations in most of the medical world. This was a real observation - nevertheless, evidence-based medicine is something very different.

FUTURE PERSPECTIVES

The room for improvement is huge in diagnostic as well as therapeutic aspects of pancreatic cancer. The development of a panel of biomarkers enabling early detection of small and localized cancerous lesions is still only a dream, but progress is speeding up, particularly the stability of free miRNA in serum[119] has fostered optimism. Even the recurrence risk after surgery and the probable response to anti-tumor therapy may be predicted and become a key to individualized treatment plans in the near future. Novel chemotherapy regimens with documented improved survival are now available[86], and even chemo- and radiotherapy resistance may be reversed through utilization of the regulatory effect of miRNA on essential molecular pathways[120].

Surgical performance has improved significantly in large volume centers and the laparoscopic technique is well established for distal resections[121,122]. Skepticism remains for laparoscopic resection of adenocarcinoma but the rate of R0 resection was 91% and five year survival 30% in a recent report[34]. Accordingly, oncological results

are equal or may even be better after laparoscopic than open resection. Also pancreaticoduodenectomy (Whipple-procedures) may be performed laparoscopically, but available data on outcome are scarce[123]. Robotic surgery might generate security advantages in this field[124], and it seems reasonable to assume that the immunosuppressive effect of surgery can be significantly reduced when an open Whipple-procedure is replaced by a laparoscopic operation. This might represent a greater window of opportunity for adjuvant immunotherapy, becoming more effective when inhibitory immunoregulation is downgraded or even eliminated.

The need of well-designed prospective trials clarifying the role of neoadjuvant chemotherapy is underlined also by other authors[125,126]. Important standardization of staging and treatment is incorporated in the Intergroup trial (Alliance A021101)[79], which is conducted as a single arm pilot study, intended to serve as paradigm for future randomized comparative trials.

CONCLUSION

Curative treatment outcome for patients with pancreatic cancer is achievable if early surgical treatment is combined with adjuvant chemotherapy. Nevertheless, most patients end up in a palliative situation, earlier or later. Also palliative therapeutic interventions are improving, but a multidisciplinary team with advanced expertise is a prerequisite for optimal care. Translational research is the key to personalized treatment plans, which is strongly needed in patients with pancreatic cancer[127].

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2014 ADVANCES IN PANCREATIC CANCER

Involvement of eicosanoids in the pathogenesis of pancreatic cancer: The roles of cyclooxygenase-2 and 5-lipoxygenase

Lawrence M Knab, Paul J Grippo, David J Bentrem

Lawrence M Knab, David J Bentrem, Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, United States
Paul J Grippo, David J Bentrem, Robert H Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL 60611, United States
David J Bentrem, Jesse Brown VA Medical Center, Chicago, IL 60612, United States
Author contributions: Knab LM, Grippo PJ and Bentrem DJ developed topic selection; Knab LM wrote the paper; Grippo PJ and Bentrem DJ revised the paper.
Correspondence to: David J Bentrem, MD, Department of Surgery, Northwestern University Feinberg School of Medicine, Northwestern University, Suite 650, 676 N St. Clair, Chicago, IL 60611, United States. dbentrem@nmff.org
Telephone: +1-312-6954113 Fax: +1-312-6951462
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The mechanism of COX-2 has been shown to include effects on apoptosis as well as angiogenesis. 5-LOX has been implicated in apoptosis. The use of COX-2 and 5-LOX inhibitors in clinical studies in patients with pancreatic cancer has been limited. Patient enrollment has been restricted to those with advanced disease which makes evaluation of these drugs as chemopreventive agents difficult. COX-2 and 5-LOX expression have been shown to be present during the early neoplastic changes of pancreatic cancer, well before progression to invasive disease. This indicates that the ideal role for these interventions is early in the disease process as preventive agents, perhaps in patients with chronic pancreatitis or hereditary pancreatitis.

Core tip: This review article highlights the relationship between inflammation and pancreatic cancer, specifically focusing on the enzymes cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX). The role of inflammation and tumor progression is a burgeoning area of research. This review delves into the research that has been conducted investigating COX-2 and 5-LOX and their relationship to pancreatic cancer both *in vivo* and *in vitro*. We discuss a variety of investigations including basic science, epidemiological, and clinical as they relate to pancreatic inflammation and eicosanoids.

Original sources: Knab LM, Grippo PJ, Bentrem DJ. Involvement of eicosanoids in the pathogenesis of pancreatic cancer: The roles of cyclooxygenase-2 and 5-lipoxygenase. *World J Gastroenterol* 2014; 20(31): 10729-10739 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i31/10729.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i31.10729

Abstract

The interplay between inflammation and cancer progression is a growing area of research. A combination of clinical, epidemiological, and basic science investigations indicate that there is a relationship between inflammatory changes in the pancreas and neoplastic progression. Diets high in ω-6 polyunsaturated fatty acids provide increased substrate for arachidonic acid metabolism by cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) to form eicosanoids. These eicosanoids directly contribute to pancreatic cancer cell proliferation. Both COX-2 and 5-LOX are upregulated in multiple cancer types, including pancreatic cancer. *In vitro* studies using pancreatic cancer cell lines have demonstrated upregulation of COX-2 and 5-LOX at both the mRNA and protein levels. When COX-2 and 5-LOX are blocked *via* a variety of mechanisms, cancer cell proliferation is abrogated both *in vitro* and *in vivo*.

INTRODUCTION

The relationship between inflammation and cancer is well established. Rudolf Virchow noticed leukocytes in cancerous tissue as early as 1863 and conjectured that there was a link between chronic inflammation and neoplasia[1]. This theory has been validated by clinical examples such as Marjolin's ulcers which are squamous cell carcinomas that form in sites of chronic inflammation such as burn scars or chronic ulcers[2]. Other examples of inflammatory conditions with correlative cancers are inflammatory bowel disease and colorectal cancer, gastritis caused by *Helicobacter pylori* and gastric cancer, hepatitis and hepatocellular carcinoma, and chronic pancreatitis and pancreatic cancer. These examples highlight the impact of inflammation on the neoplastic process though the mechanism is unclear.

The inflammatory response is marked by cytokine release from epithelial cells which attract and activate inflammatory cells. When macrophages, neutrophils, fibroblasts, and mast cells are attracted to this inflammatory microenvironment, they produce reactive oxygen species (ROS) and stimulate epithelial cell proliferation[3]. The infiltration of these cells into the tumor microenvironment has been implicated in pancreatic tumor progression (Figure 1)[4-7]. ROS can directly cause DNA damage by increasing the probability that genetic mutation will occur. Combined with their effects on cellular proliferation, ROS increase the likelihood of neoplastic transformation[3,8]. A key step in the inflammatory process is the activation of the arachidonic acid pathway that produces eicosanoids. The purpose of this paper will be to review inflammatory mechanisms as they relate to pancreatic cancer, specifically the roles of cyclooxygenase (COX) and lipoxygenase (LOX), and how their metabolites contribute to carcinogenesis.

INFLAMMATION AND PANCREATIC CANCER

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, and the vast majority of those afflicted succumb to this disease. The 5-year survival rate is about 5%-6%[9]. Since the majority of pancreatic cancer is discovered late in the disease process, well after potentially curative surgery is an option, understanding the early oncogenic changes is necessary to aid in prevention. Since inflammation has been shown to be a key factor in the neoplastic process as it contributes to genetic changes and DNA damage, its role in pancreatic cancer is of particular interest.

Studying the mechanisms of pancreatitis in patients can be helpful for understanding inflammation as it relates to pancreatic cancer development. Patients with hereditary pancreatitis, a rare disease responsible for less than 1% of pancreatitis cases, have frequent episodes of acute inflammation[10]. Repeated episodes of pancreatitis result in fibrosis, chronic inflammation, and the eventual destruction of the gland[11]. This chronic inflammatory environment is thought to contribute to malignant transformation of pancreatic ductal cells. In patients with hereditary pancreatitis, the risk of developing pancreatic cancer is 53 times higher than unaffected individuals, and by 70 years of age, approximately 40% of these patients will develop pancreatic cancer[10]. Patients afflicted with non-hereditary chronic pancreatitis also have an increased risk of pancreatic cancer. Population studies suggest that patients with chronic pancreatitis are 17 times more likely to develop pancreatic cancer compared to age matched controls, and the risk is correlated with the duration of inflammation[12]. Therefore it will be important to understand the mechanisms that link pancreatitis to the development of pancreatic cancer.

The inflammatory process begins with the inappropriate release of proteolytic pancreatic enzymes that cause acinar cell injury[13]. This generates an immune response in which inflammatory cells are attracted to cytokines released from the cells at the site of injury. Our lab, as well as others, previously investigated the relationship between one of the major inflammatory cell types, mast cells, and pancreatic cancer[6,14]. We have shown that mast cell infiltration in pancreatic cancer specimens correlates with worse prognosis[6]. Ma demonstrated that pancreatic ductal adenocarcinoma (PDAC) cells promote mast cell migration and activation *in vitro*. The study also showed that blocking mast cell migration in an orthotopic PDAC mouse model decreased PDAC growth *in vivo*[15]. Similarly, Soucek demonstrated in an islet-cell tumor mouse model that mast cells mediate expansion of these tumors and are essential for tumor maintenance[5].

The generation of ROS and activation of the arachidonic acid pathway are also key steps in potentiating the inflammatory response[13]. The body mounts a natural response to chronic insults to the pancreas by releasing growth factors such as platelet-derived growth factor and transforming growth factor beta. This stimulates cell proliferation, which can potentially worsen DNA damage and increase genetic mutations[16].

EPIDEMIOLOGICAL STUDIES

Epidemiological studies have shown that high-fat diets, specifically with a high proportion of polyunsaturated omega-6 fatty acids, are associated with increased cancer rates, particularly in breast, pancreas, and prostate cancers[17-22]. Studies have shown that cancer incidence in an ethnic group often changes after migration and drastic dietary changes. An example is the migration of the Japanese to Western countries that have relatively higher fat diets compared to Japanese diets. Studies have reported increased colon, pancreas, breast, and prostate cancer incidence in individuals migrating to Western countries from Japan[21]. The relationship between a high-fat diet and pancreatic cancer was evaluated by a prospective study investigating obesity in various age groups including early adulthood, midlife, and older age. There were
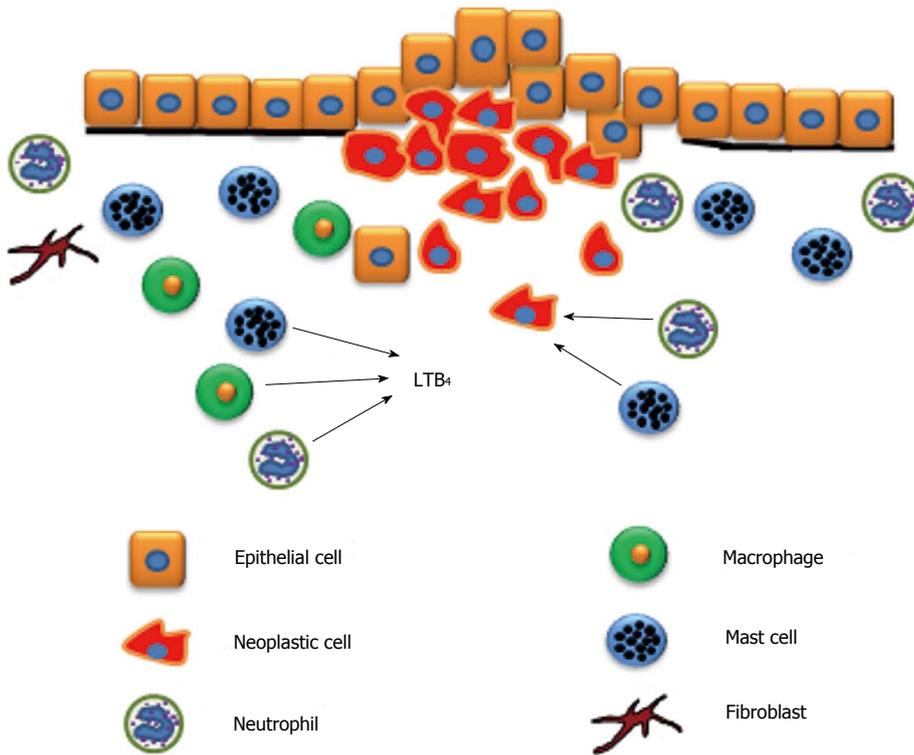
Figure 1 Inflammatory cell infiltration into the tumor microenvironment. As pancreatic adenocarcinoma progresses, inflammatory cells such as mast cells, neutrophils, and macrophages are attracted to the tumor microenvironment and enhance tumor growth. Leukotriene B4 (LTB4) is a chemotactic factor for macrophages, neutrophils, and mast cells. Fibroblasts are also activated and enhance collagen production.

significant positive associations between pancreatic cancer and obesity in all age groups studied[23]. Patients with the longest duration of obesity and diabetes were at the greatest risk for pancreatic cancer[23]. One of the mechanisms proposed for this association is the high content of arachidonic acid in animal fats. Arachidonic acid is metabolized to biologically active lipids by COX, LOX, and epoxygenase pathways to generate eicosanoids[24]. Eicosanoids have been implicated in various carcinogenic mechanisms including tumor progression and metastasis[25]. Studies conducted in EL-Kras transgenic mice fed a high ω-6 fatty acid diet demonstrated increased frequency and size of pancreatic neoplastic lesions as well as increased pancreatic mast cell densities[26]. In a related study, a high ω-3 fatty acid diet in EL-Kras transgenic mice was found to have a protective effect against the formation of pancreatic lesions. These mice had reduced incidence, frequency, and proliferative index of pancreatic precancer compared to those fed standard chow[27]. In unpublished findings by our lab, we demonstrate that EL-Kras transgenic mice fed high ω-6 fatty acid diets had increased PGE2 and LTB4 compared to their counterparts fed a high ω-3 fatty acid diet. Therefore, ω-3 and ω-6 fatty acids are involved in carcinogenic mechanisms and have opposing effects on pancreatic neoplasia, which is hypothesized to be mediated through the regulation of eicosanoid production.

Further evidence to support the role of eicosanoids in the carcinogenic process are epidemiological studies indicating that the use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of various solid tumors[24,28]. One study used a meta-analysis to examine the effect of regular NSAID use on colon, lung, breast, and prostate cancers. The results indicated that there is a risk reduction of 43% for colon cancer, 28% for lung cancer, 25% for breast cancer, and 27% for prostate cancer[28]. The role of NSAIDs and pancreatic cancer is not clear. Anderson conducted a prospective study with 28000 post-menopausal women and demonstrated a decreasing trend in pancreatic cancer incidence in women with more frequent aspirin use[29]. Alternatively, a study among United States adults followed for 18 years found no association between aspirin use and pancreatic cancer mortality[30]. A different prospective study in a large cohort of women with an 18 year follow-up showed an association with long-term aspirin use and pancreatic cancer although there was a higher prevalence of obesity and diabetes mellitus among patients who reported regular aspirin use[31]. A study conducted in the United Kingdom demonstrated that NSAID use for more than 773 d in the 5 years prior to diagnosis was associated with a 20% risk reduction of pancreatic cancer, although increasing doses did not have an impact on risk[32]. A meta-analysis involving 11 studies analyzing the association between pancreatic cancer and aspirin and other NSAIDS did not find a conclusive association[33]. The summary relative risk did not find an association between aspirin or other NSAIDS and pancreatic cancer, nor an association between regular use vs irregular use, nor frequency of aspirin or NSAID use[33].
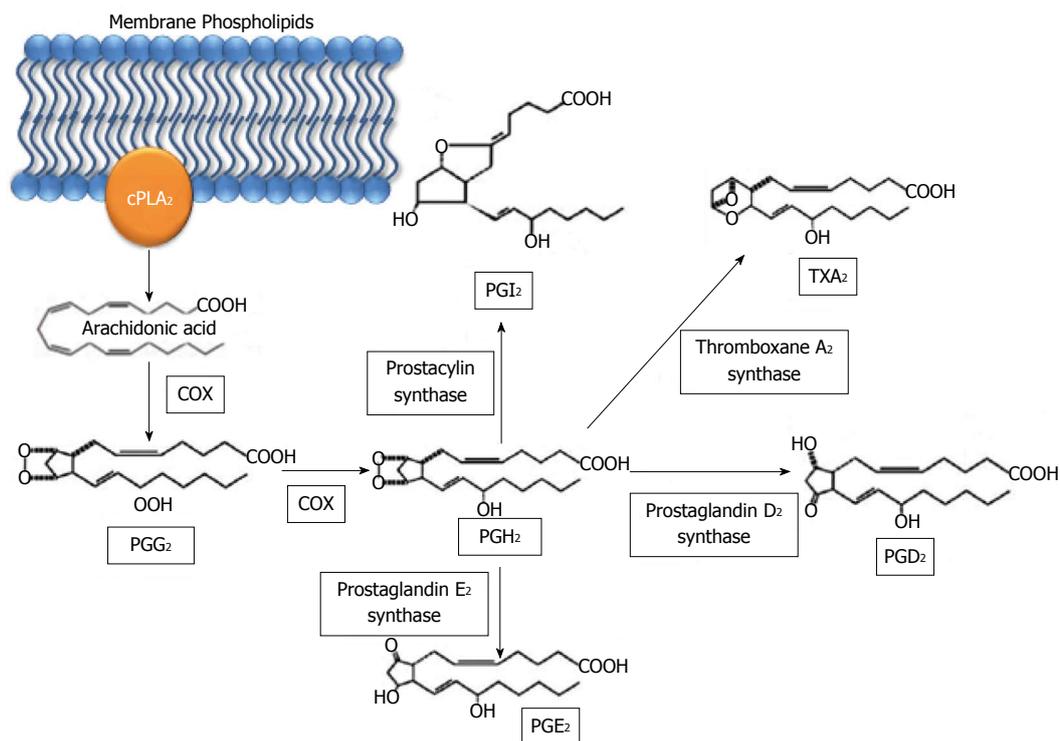
Figure 2 Metabolic pathway of prostaglandins *via* cyclooxygenase. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and converted to PGG_2 and subsequently PGH_2 by COX. PGH_2 is then converted to PGI_2, TXA_2, PGD_2, and PGE_2. cPLA2: Cytosolic phospholipase A_2; COX: Cyclooxygenase; PG: Prostaglandin; TX: Thromboxane.

BIOCHEMISTRY OF COX AND LOX

The ability of NSAIDs to exert their anti-inflammatory and anti-tumor effects by inhibiting the COX enzyme, which results in decreased prostanoid production, demonstrates the intimate relationship between inflammation and cancer[24]. There is evidence to suggest that 5-LOX, a close relative of COX-2, is essential for eicosanoid production and tumor pathogenesis. The precursors of eicosanoids are arachidonic acids. Both prostaglandins (PG) and leukotrienes (LT) are members of the eicosanoid family, which are lipid mediators made of a 20 carbon fatty acid derivative[34]. Eicosanoids are vital due to their distinct biological activity in the body and effectiveness in the nanomolar concentration range[34]. The two eicosanoid members that will be discussed in detail here are prostaglandins and leukotrienes.

Prostaglandins are made by most cells in the body, and they act as both paracrine and autocrine mediators[34]. Arachidonic acid is released from the membrane by the phospholipase cPLA2 and acted on by prostaglandin G/H synthase (known as COX) to become an intermediate known as PGH_2[24] (Figure 2). There are two main forms of COX: COX-1 and COX-2. COX-1 is generally thought of as the constitutively expressed enzyme that is responsible for basal production of prostanoids for tissue homeostasis, and COX-2 is induced by cytokines and growth factors, particularly at sites of inflammation and neoplasia[13]. Therefore, COX-2 has a key role in the setting of inflammation and the tumor microenvironment[24].

Leukotrienes, while derived from the same precursor as prostaglandins, are functionally distinct. Leukotrienes are predominately produced by inflammatory cells, and once cellular activation occurs, cPLA2 and 5-lipoxygenase (5-LOX) are translocated to the nuclear envelope[34]. LOX enzymes are a family of nonheme iron-containing dioxygenases with labeling based on the location of oxygen insertion at the carbon position of arachidonic acid[25]. The most common LOX enzymes are 5-, 8-, 12-, and 15-LOX[25]. These then form the corresponding hydroperoxyeicosatetraenoic acids (HPETE)[25]. Specifically, 5-LOX transforms arachidonic acid *via* a dehydration reaction to the unstable epoxide LTA_4[25]. LTA_4 is further oxidized to form either 5-HETE or the leukotrienes[25]. LTA_4 can be hydrolyzed by leukotriene A4 hydrolase in the cytoplasm or nucleus resulting in LTB_4 (Figure 3). LTB_4 is known as a potent chemoattractant, and its receptors are upregulated in pancreatic cancer[35]. LTA_4 can also be conjugated with glutathione to form LTC_4 by LTC_4 synthase. LTC_4 can then undergo extracellular metabolism resulting in LTD_4 and LTE_4[34]. The activation of 5-LOX is dependent upon the 5-LOX-activating protein (FLAP).

One of the ways in which LTB_4 directs chemotaxis and regulates neutrophil adhesion is by activating integrin receptors[34,36-39]. It has been demonstrated that local cell death causes "swarm-like" interstitial neutrophil clustering and LTB_4 plays an important role in intercellular communication between neutrophils and facilitates neu-
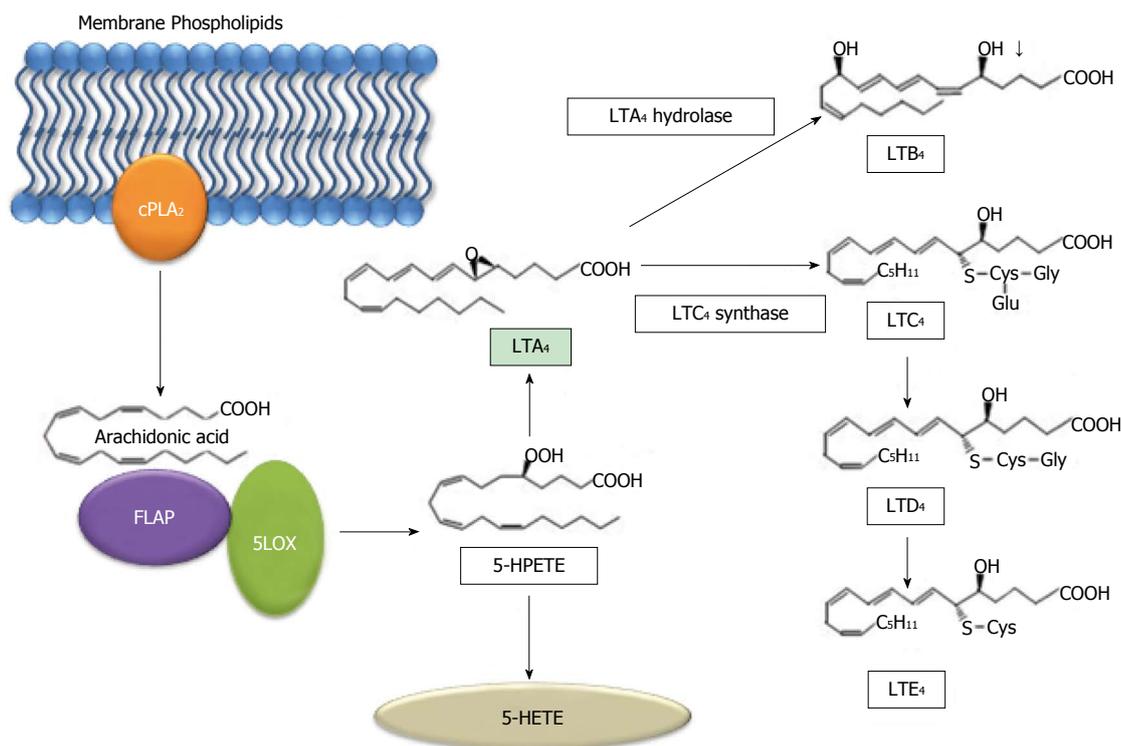
Figure 3 Metabolic pathway of arachidonic acid *via* 5- lipoxygenase. Arachidonic acid is released from membrane phospholipids by phospholipase A_2 and converted to 5-HPETE by 5-LOX and 5-LOX activating protein (FLAP). 5-HPETE can then form either 5-HETE or LTA_4. LTA_4 then becomes LTB_4 or LTC_4. LTC_4 can then form LTD_4 and subsequently LTE_4. cPLA2: Cytosolic phospholipase A_2; LOX: Lipoxygenase; FLAP: 5-LOX activating protein; HPETE: Hydroperoxyeicosatetraenoic acid; HETE: Hydroxyl 6 *trans* 8, 11, 14 *cis* eicosatetraenoic acid; LT: Leukotriene.

trophil movement through tissue[39]. In the tumor microenvironment, LTB_4 has been shown *in vivo* to enhance leukocyte recruitment into the tumor stroma[40].

ROLE OF COX IN PANCREATIC NEOPLASIA AND CANCER

COX-2 expression is upregulated in a variety of malignancies including colon, esophagus, breast, and pancreatic cancer[41-43]. Multiple studies have indicated that COX-2 is also important in carcinogenesis. One example in a murine model of familial adenomatous polyposis showed a marked reduction in the number and size of intestinal polyps in COX-2 null mice with an APC mutation[44].

The relationship between COX-2 and pancreatic cancer has been evaluated in multiple studies with the majority of the evidence demonstrating upregulated COX-2 expression in pancreatic cancer at both the mRNA and protein levels. One study showed that levels of COX-2 mRNA were increased 60-fold in pancreatic cancer compared to normal tissue. In addition, COX-2 protein was expressed in 9 out of 10 pancreatic cancer samples, while nontumor samples had no COX-2 expression[45]. Immunohistochemistry (IHC) confirmed COX-2 expression in malignant epithelial cells[45]. A different study demonstrated an increase in COX-2 expression using IHC when pancreatic carcinoma was compared to normal pancreas[43]. Five pancreatic cancer cell lines were

studied, and COX-2 protein expression was detected in BxPC-3, Capan-1, and MDAPanc-3 cells, and increased levels of COX-2 mRNA were detected in 4 of the 5 cell lines[43]. When an NSAID was used, a dose-dependent inhibition of cellular proliferation was observed in all cell lines studied[43]. Kokawa *et al*[46] used different pancreatic cancer cell lines (KP-2, PNS-1, MiaPaca-2, and Panc-1) to show that COX-2 expression was upregulated in all 4 of them, and NSAID inhibition of cellular proliferation was correlated with the expression of COX-2. Maitra used automated cellular imaging to evaluate COX-2 expression not only in pancreatic adenocarcinoma but also its precursor, pancreatic intraepithelial neoplasia (PanIN). This showed an increase in the overall average number of positive cells from 19.2% in normal ducts to 36.3% in PanINs to 47.3% in adenocarcinomas[47]. This study suggests tumorigenic activity of COX-2 in preinvasive pancreatic lesions and a potential role for chemopreventive agents such as COX-2 inhibitors in pancreatic cancer.

While multiple studies have shown the association between pancreatic cancer and COX-2 expression, few have investigated the underlying mechanism of COX-2 and how it promotes neoplastic changes. Overexpression of COX-2 leads to increased tumor prostanoid levels, and PGE_2 is known to have several tumorigenic effects. PGE_2 has been implicated in the inhibition of apoptosis and the induction of proliferation and angiogenesis[48]. One group investigated the relationship between high-mobility group A1 (HMGA1) and COX-2 in pancreatic

cancer. The authors proposed that the HMGA1-COX-2 axis is a key molecular pathway in pancreatic cancer because the upregulation of COX-2 expression is HMGA1 dependent in various pancreatic cancer cell lines. It was first demonstrated that a positive correlation between HMGA1 and COX-2 expression in six pancreatic cancer cell lines (BxPC-3, HPAF-II, MiaPaCa-2, Panc1, PL45, and XPA-3) existed[49]. COX-2 expression after knockdown of HMGA1 in two pancreatic cancer cell lines was evaluated and showed that HMGA1 binds to the COX-2 promoter to induce its expression[49]. A significant reduction in COX-2 expression after using an HMGA1 siRNA was observed, and COX-2 inhibitors blocked tumorigenesis in human pancreatic cancer xenografts that overexpressed HMGA1[49].

Another potential mechanism proposed for the involvement of COX-2 in tumorigenesis is its effect on angiogenesis. Chu compared the angiogenic effects of a COX-2 expressing pancreatic cancer cell line BxPC-3 with the COX-2 negative AsPC-1 cell line. The group found a significant increase in endothelial cell migration induced by BxPC-3 migration compared with AsPC-1. These findings were supported by data demonstrating that BxPC-3 treatment with a COX-2 inhibitor decreased the angiogenic responses of the endothelial cells[50]. Eibl *et al*[51] showed in a subset of pancreatic cancer cell lines that COX-2 increased PGE2 which subsequently increased VEGF secretion. In a subsequent *in vivo* study, an orthotopic pancreatic cancer model in nude mice was used to demonstrate the effects of nimesulide, a selective COX-2 inhibitor, on angiogenesis. In mice with COX-2 positive tumors, nimesulide resulted in an increase in VEGF production by malignant cells but a compensatory decrease in production by nonmalignant cells, ultimately leading to reduced tumor angiogenesis and growth[52].

Ito's study on the effect of COX-2 on tumor invasion found that PGE2 mediated pancreatic cancer cell invasion through induction of matrix metalloproteinase-2 expression. This induction was found to be dependent on an extracellular signal-regulated kinase (ERK)/Ets-1-dependent mechanism[53].

Another study investigated the expression of COX-2 on clinical outcomes and found no correlation between global COX-2 expression and clinical outcome. The clinical outcomes studied were survival, stage, tumor size, or vascular invasion[54]. The expression of COX-2 was related to an increase in perineural invasion[54].

Several preclinical mouse models evaluating pancreatic lesions have been reported. One particular transgenic model, LSL-KRASG12D; PDX-1-Cre, is a mouse with a KRAS mutation expressed in pancreatic progenitor cells. This model results in PanIN lesions which eventually develop through advanced PanIN lesions into adenocarcinoma[55]. The efficacy of a selective COX-2 inhibitor, nimesulide, was evaluated in this mouse model. Animals treated with nimesulide demonstrated significantly fewer PanIN lesions and decreased intrapancreatic prostaglandin E2 levels compared to mice on a control diet[55].

In two unpublished works from our group, another mouse model with mutant Kras expression targeted to acinar cells (EL-Kras)[56] have been crossed with COX-2 knock-out mice to generate cohorts of EL-Kras/COX-2-/- mice. These mice have a significantly reduced frequency of cystic papillary neoplasms compared with EL-Kras mice with wild-type COX-2. Also, mice that overexpress COX-2 in acinar cells develop hyperplastic, mildly dysplastic ducts with accompanying focal fibrosis and lymphocytic infiltration[57]. A different transgenic mouse model, BK5.COX2, results in COX-2 overexpression in the exocrine pancreas[58]. The resulting histology demonstrated pancreatitis-like changes with acinar-to-ductal metaplasia by 3 mo, and at 6-8 mo strongly dysplastic features. The described phenotype was completely prevented by maintaining the mice on a COX-2 inhibitor. Cell lines derived from lesions in these mice were tumorigenic when injected into nude mice. Both of these mouse models highlight the relationship between COX-2 and pancreatic cancer and will be important in future studies.

ROLE OF LOX IN PANCREATIC NEOPLASIA AND CANCER

Similar to COX-2, LOX has been implicated in several human cancers including lung, prostate, colon, breast, and pancreatic; however, relatively little research has been conducted to elucidate its role in cancer progression[59-61]. 5-LOX expression is upregulated in both pancreatic adenocarcinoma as well as in neoplastic lesions of the pancreas[25]. In a study by Hennig, three pancreatic cancer cell lines, AsPC-1, PANC-1, and MiaPaCa2, were found to have 5-LOX mRNA expression while normal human pancreatic cells did not express 5-LOX[35]. They also confirmed that 5-LOX protein was expressed in these cell lines and in two additional cell lines, Capan-1 and HPAF[35]. Moreover, the expression levels of both 5-LOX and its downstream metabolite LTB4 were found to be significantly upregulated in pancreatic tumors compared with normal pancreatic tissue[35]. Interestingly, staining was evident in both the cancer cells as well as the ductal cells and adjacent islets. A follow-up study by Hennig *et al*[62] investigated 5-LOX expression in PanIN lesions. Greater than 90% of the ductal cells had strong positive 5-LOX staining in all grades of PanINs with no significant difference between grades of PanINs. This was compared to normal pancreatic specimens that had 0 to 7.5% of the ductal cells showing 5-LOX staining[62]. This study also reported that 5-LOX expression was present in pancreatic PanIN-like lesions in N-nitroso-bis (2-oxopropyl)-amine (BOP) treated hamsters as well as EL-Kras transgenic mice[62]. Ding reported similar results showing increased 5-LOX expression in MiaPaCa2, PANC-1, AsPC-1, and Capan2 pancreatic cancer cell lines at the mRNA level[63]. The general LOX inhibitor (NDGA), a 5-LOX inhibitor (Rev5901), and a FLAP inhibitor (MK-886), all inhibited thymidine incorpora-

tion in MiaPaCa2 cells indicating that these compounds induced growth inhibition in pancreatic cancer cells. Finally, it was demonstrated that arachidonic acid and linoleic acid induced pancreatic cancer cell proliferation[63].

While there have been no studies published to date examining mouse models deficient in 5-LOX, our lab is currently investigating this mouse model. We have developed a EL-Kras/5-LOX null mouse and preliminary results have indicated a decrease in pancreatic lesions in the 5-LOX null mice compared with their wildtype counterparts.

While it is well established that 5-LOX plays an important role in pancreatic tumor progression, fewer studies have investigated its underlying mechanism in this disease. Ding showed that the 5-LOX metabolite, 5(S)-hydroxyeicosatetraenoic acid [5(S)-HETE], stimulates pancreatic cancer cell proliferation in a time- and concentration-dependent manner[63]. In a subsequent study, Ding demonstrated that 5-(S)-HETE has mitogenic effects due to its role in the MEK/ERK and PI3 kinase/AKT pathways[64]. In an additional study, this group demonstrated that both the general LOX inhibitor (NDGA) and the 5-LOX inhibitor (Rev5901) induced apoptosis in four different pancreatic cancer cell lines[65]. Apoptosis was confirmed using three different methods including DNA propidium iodide staining, DNA fragmentation, and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay in PANC-1, MiaPaCa2, Capan2, and HPAF cell lines[65]. A follow-up study performed by Tong further delineated the mechanism behind the LOX inhibitor-induced apoptosis showing that it is a mitochondria-mediated pathway[66]. Specifically, LOX inhibitors (NDGA and Rev5901) decreased Bcl-2 and Mcl-1 and increased Bax expression in human pancreatic cancer cells[66]. LOX inhibitors also induced cytochrome-c release and caspase-9 activation. The effect of the LOX inhibitors was also demonstrated *in vivo* where it blocked pancreatic cancer cell growth and induced apoptosis in athymic mice[66]. These studies suggest the relationship between 5-LOX and its role in apoptosis in the tumor microenvironment.

LTB4 AND PANCREATIC CANCER

LTB4 is a metabolite of 5-LOX and an important inflammatory mediator. LTB4 is involved in recruiting inflammatory cells and is a potent chemokine for monocytes, neutrophils, and eosinophils. It also enhances adhesion and migration of neutrophils across the vascular endothelium[67]. BLT1 and BLT2 are two G-protein-coupled receptors that have a high and low affinity, respectively, for LTB4[68]. LTB4 is secreted from human pancreatic cancer cells and its receptors are upregulated in pancreatic cancer tissue as well as in multiple cell lines[35,69]. Similar to COX-2 and 5-LOX, BLT1 and BLT2 have also been found to be upregulated in PanIN lesions which suggests a potential role of LTB4 and its receptors in chemoprevention[70].

Multiple LTB4 receptor antagonists have been developed but earlier compounds had poor oral bioavailability[68]. A more stable and orally bioavailable compound was later developed, LY293111, which blocks LTB4-mediated kinase phosphorylation[67]. LY293111 inhibits pancreatic cancer growth *in vivo* and *in vitro* through inhibition of proliferation and induction of apoptosis in a variety of pancreatic cancer cell lines (MiaPaCa-2, HPAC, Capan-1, Capan-2, PANC-1, and AsPC-1) in a time- and concentration-dependent manner[69,71]. When LTB4 was added to the cancer cell lines, it stimulated proliferation and induced ERK1/2 phosphorylation in all six cell lines[69]. In a different study, LY293111 was found to cause cell cycle arrest in S phase and suppress cyclin A, cyclin E, and cdk2 expression[71]. When LY293111 was administered to athymic mice with human pancreatic cancer xenografts, the LTB4 receptor antagonist suppressed growth of the subcutaneous xenografts[69].

CLINICAL CORRELATION

COX inhibitors

Multiple studies have been conducted evaluating the use of COX-2 inhibitors combined with different chemotherapy regimens. A phase II trial of Uracil/Tegafur plus Leucovorin and Celecoxib combined with radiotherapy in patients with locally advanced pancreatic cancer did not show a significant response and resulted in substantial gastrointestinal toxicity[72]. A study of Celecoxib and 5-fluorouracil in patients with advanced pancreatic cancer who had progressed after gemcitabine-based chemotherapy showed promising results in that the Celecoxib was well tolerated and capable of inducing durable responses[73]. In a phase II trial of gemcitabine, Irinotecan, and Celecoxib in patients with inoperable pancreatic cancer, the addition of Celecoxib was found to increase the percentage of patients achieving a one-year overall survival from about 3 mo to 9 mo and increased overall survival from about 6 mo to 18 mo[74]. Other studies in patients with advanced pancreatic cancer evaluated the combination of Celecoxib and gemcitabine or the combination of Gemcitabine, Celecoxib, and Cisplatin, but Celecoxib did not increase the efficacy of either chemotherapy regimen (Table 1)[75,76]. While the idea of using a COX-2 inhibitor is promising in patients with pancreatic cancer, it will likely be most effective as a preventive agent very early in the disease process as opposed to improving survival in those patients with advanced disease.

LOX inhibitors

Zileuton is a 5-LOX inhibitor of the N-hydroxyurea series, approved by the Food and Drug Administration in 1996 for the treatment of asthma[25]. It was shown in clinical trials to produce moderate airway improvement in asthmatics. While Zileuton has had promising effects for airway disease, this drug has not yet been tested in patients with cancer.

Several studies have investigated Zileuton in animal

Table 1 Clinical trials						
Drug	**Type**	**Trial**	**Type**	**Cancer**	**Outcome**	**Toxicity**
Celecoxib	COX-2 inhibitor	Uracil/Tegafur, Leucovorin, Celecoxib + RT[72]	II	Pancreatic; locally advanced unresectable	No significant partial or complete response	Significant GI toxicity
		Celecoxib, 5-FU[73]	Pilot study	Pancreatic; advanced after Gemcitabine treatment	Durable response	Well tolerated
		Gemcitabine, irinotecan, celecoxib[74]	II	Pancreatic; unresectable	Increased OS from 6 m to 18 m	Well tolerated
		Gemcitabine, celecoxib[76]	II	Pancreatic; locally advanced or metastatic	No significant response	Well tolerated
		Gemcitabine, cisplatin, celecoxib[75]	II	Pancreatic; metastatic	No significant response	Well tolerated
LY293111	LTB4 receptor antagonist	Irinotecan, LY293111[80]	I	Solid tumors (including pancreatic); locally advanced or metastatic	No significant response	Significant GI toxicity
		Gemcitabine, LY293111[81]	II	Pancreatic; locally advanced or metastatic	No significant response	Significant GI toxicity

Cox: Cyclooxygenase; LTB4: Leukotriene B4; RT: Radiation therapy; FU: Fluorouracil; OS: Overall survival; GI: Gastrointestinal.

studies and shown promising results for multiple cancers including carcinoma of the colon, lung, and pancreas. Zileuton was shown to reduce cell proliferation in murine colon adenocarcinoma cell lines[77]. In a xenograft model using human colon cancer cells, Zileuton inhibited tumor growth and reduced tumor mass[78]. In pancreatic cancer studies using the Syrian hamster model with BOP-induced pancreatic cancer, Zyflo (an extended release formulation of Zileuton) was found to reduce the incidence and size of the pancreatic cancer both alone and in combination with a COX-2 inhibitor[79].

LTB4 RECEPTOR ANTAGONIST

A few clinical trials have been conducted using LY293111 in patients with pancreatic cancer. A phase I study demonstrated that LY29311 was well tolerated in combination with Irinotecan although no responses were seen[80]. A different study randomized patients with pancreatic cancer to gemcitabine and LY293111 *vs* gemcitabine and placebo. There was no significant difference in six-month survival or progression-free survival[81]. Finally, a study conducted in patients with non-small cell lung cancer receiving LY293111 and Cisplatin/Gemcitabine also did not show a survival benefit[82]. Similar to COX-2 inhibitors, an LTB4 receptor antagonist would probably be most efficacious early in the disease process.

FLAP inhibitors

MK-886 is a FLAP inhibitor and inhibits leukotriene biosynthesis. It was first developed for use in asthma although clinical development was halted due to only a 50% inhibition of leukotriene production when used[83]. A second-generation FLAP inhibitor, MK-0591, had more potent inhibitory effects on leukotriene production, although it did not clinically perform as expected and was also discontinued[84].

Similar to Zileuton, MK-886 has shown promising results *in vitro* and *in vivo*. As mentioned above, MK-866 was shown to promote growth inhibition in a pancreatic

cancer cell line. It was also shown *in vivo* to reduce pancreatic cancer development in a hamster model[85].

CONCLUSION

The inflammatory pathway is an important process in cancer progression. A combination of clinical studies, epidemiological studies, and basic science investigations indicate that there is a relationship between inflammatory changes in the pancreas and neoplastic progression. Intake of ω-6 polyunsaturated fatty acids provides increased substrate for COX and LOX mediated metabolism of arachidonic acid into eicosanoids. These eicosanoids directly contribute to pancreatic cancer cell proliferation. When COX-2 and 5-LOX are blocked *via* a variety of mechanisms, cancer cell proliferation is abrogated both *in vitro* and *in vivo*. The use of COX-2 and 5-LOX inhibitors in clinical studies in patients with pancreatic cancer has been limited. Patient enrollment has been restricted to patients with advanced disease which makes evaluation of these drugs as chemopreventive agents difficult. COX and LOX expression have been shown to be present during the early neoplastic changes of pancreatic cancer, well before progression to invasive disease. This indicates that the ideal role for these interventions is early in the disease process as preventive agents, perhaps in patients with chronic pancreatitis or hereditary pancreatitis. Further investigation is needed to broaden our understanding of the complex relationship between inflammation and pancreatic cancer and how these inflammatory pathways can be targeted to treat this deadly disease.

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2014 ADVANCES IN PANCREATIC CANCER

WJG 20ᵗʰ Anniversary Special Issues (14): Pancreatic cancer

Borderline resectable pancreatic cancer: Definitions and management

Nicole E Lopez, Cristina Prendergast, Andrew M Lowy

Nicole E Lopez, Cristina Prendergast, Andrew M Lowy, Division of Surgical Oncology, Department of Surgery, Moores Cancer Center, University of California San Diego, La Jolla, CA 92093-0987, United States

Author contributions: All the authors contributed equally to this work.

Correspondence to: Andrew M Lowy, MD, Division of Surgical Oncology, Department of Surgery, Moores Cancer Center, University of California San Diego, 3855 Health Sciences Drive #0987, La Jolla, CA 92093-0987, United States. alowy@ucsd.edu

Telephone: +1-858-8222124 Fax: +1-858-5344813

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Abstract

Pancreatic cancer is the fourth leading cause of cancer death in the United States. While surgical resection remains the only curative option, more than 80% of patients present with unresectable disease. Unfortunately, even among those who undergo resection, the reported median survival is 15-23 mo, with a 5-year survival of approximately 20%. Disappointingly, over the past several decades, despite improvements in diagnostic imaging, surgical technique and chemotherapeutic options, only modest improvements in survival have been realized. Nevertheless, it remains clear that surgical resection is a prerequisite for achieving long-term survival and cure. There is now emerging consensus that a subgroup of patients, previously considered poor candidates for resection because of the relationship of their primary tumor to surrounding vasculature, may benefit from resection, particularly when preceded by neoadjuvant therapy. This stage of disease, termed borderline resectable pancreatic cancer, has become of increasing interest and is now the focus of a multi-institutional clinical trial. Here we outline the history, progress, current treatment recommendations, and future directions for research in borderline resectable pancreatic cancer.

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Core tip: Borderline resectable pancreatic cancer has become recognized as a clinical entity worthy of study based on a number of clinical observations that recognize a continuum between resectable and locally advanced unresectable disease. There are few prospective trials and therefore no data to support specific treatment regimens in borderline resectable pancreatic ductal adenocarcinoma (PDAC). Difficulties in achieving a consensus, objective definition, small numbers of patients and variability in therapeutic algorithms have delayed progress in establishing strong evidence-based practices for diagnosis and treatment. The Alliance trial represents a first step in establishing reproducible standards by which future trials in borderline resectable PDAC can abide.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States[1]. While surgical resection remains the only curative option, more than 80% of patients present with unresectable disease[1,2]. Unfortunately, even among those who undergo resection, the reported median survival is 15-23 mo, with a 5-year survival of ap-

proximately 20%[3-5]. Disappointingly, over the past several decades, despite improvements in diagnostic imaging, surgical technique and chemotherapeutic options, only modest improvements in survival have been realized. Nevertheless, it remains clear that surgical resection is a prerequisite for achieving long-term survival and cure. There is now emerging consensus that a subgroup of patients, previously considered poor candidates for resection because of the relationship of their primary tumor to surrounding vasculature, may benefit from resection, particularly when preceded by neoadjuvant therapy.

This stage of disease, termed borderline resectable pancreatic cancer, has become of increasing interest and is now the focus of a multi-institutional clinical trial. Here we outline the history, progress, current treatment recommendations, and future directions for research in borderline resectable pancreatic cancer.

EVOLUTION OF THE BORDERLINE RESECTABLE CONCEPT

The concept of borderline resectable pancreatic cancer has evolved from several clinical observations made over decades. It has been recognized for some time that the prognosis for patients undergoing surgical resection for pancreatic ductal adenocarcinoma (PDAC) is highly dependent on margin status, with total gross excision and histologically negative margins (R0 resection) being associated with the best outcomes. Survival for patients who undergo total gross excision but have histologically positive margins (R1 resection) have a reduced survival in most series[3,6-9]. Most significantly, patient who undergo resection with residual gross tumor (R2 resection) have a prognosis similar to patients treated with non-operative therapy[9-12]. Historically, resectability of pancreatic cancer was defined by absence of distant metastases, absence of local tumor extension to the celiac axis and hepatic artery, as well as the lack of involvement of the superior mesenteric vasculature. However, data emerging in the 1990's suggested that vein resection with negative margins was associated with equivalent survival to standard PD, leading to an increasing acceptance of vascular resection (VR) in curative resections. In 1994, Allema *et al*[13] published a series of 20 superior mesenteric vein/portal vein (SMV/PV) resections, showing no significant differences in survival in comparison to standard PD and confirming both the feasibility of the procedure and the capacity to obtain R0 resections with this technique. In a similarly sized study, Fuhrman *et al*[14] confirmed the findings, concluding that vascular resection is a safe and effective means by which to attain complete resection in cases of tumor adherence to the SMV or SMV/PV confluence. In the ensuing years, others strengthened the notion that appropriately selected patients could undergo vascular resection to achieve survival outcomes similar to patients undergoing standard PD and superior to outcomes of locally advanced disease treated non-operatively[15,16]. In 2004, a group from MD Anderson reviewed all patients who

underwent PD at their institution between 1990 and 2002 to examine the effect of vascular resection on margin status and survival in PDAC[16]. Of 291 patients who underwent PD for PDAC, 181 had a standard PD and 110 had PD with vascular resection. Median survival was 26.5 mo in the standard PD group and 23.4 mo in the group that required VR ($P = 0.18$). Clearly, the extent of venous involvement has a direct relationship to operability and to final margin status. As tumors encroach on the left side of the SMV-portal vein, they encroach increasingly on the SMA. Lu *et al*[17] reported that tumor involvement of greater than half the circumference was highly specific for unresectable disease. The Ishikawa classification, established by Ishikawa *et al* in 1992, is based on radiographic findings that demonstrate the relationship of the tumor to the SMV-PV (1) normal; (2) smooth shift without narrowing; (3) unilateral narrowing; (4) bilateral narrowing; and (5) bilateral narrowing and the presence of collateral veins (Figure 1). This classification has also been used to report the relationship between SMV-PV appearance by cross-sectional imaging and prognosis.

In the early 1990s a small study was conducted in which 28 patients with localized PDAC underwent treatment with preoperative chemoradiation with 5-Fluorouracil (5-FU). After restaging, 17 out of 28 were able to undergo successful resection with few complications, confirming the feasibility and safety of neoadjuvant therapy followed by resection[18]. Similarly, a 1997 study comparing pre-operative and post-operative chemoradiation in 142 patients with resectable disease found preoperative chemoradiation offered comparable benefits to post-operative therapy and is not hindered by postoperative complications or prolonged recovery[19]. Pisters *et al*[20] found additional advantages of neoadjuvant chemoradiation with 5-FU in 35 patients with resectable PDAC. Among 20 patients who underwent resection, median survival was 25 mo, while median survival among 15 patients who did not undergo PD was 7 mo. They concluded that neoadjuvant chemoradiation results in minimal toxicity while maximizing the number of patients who get combined modality treatment and limiting PD to those most likely to benefit.

Several studies have suggested that neoadjuvant chemoradiation may enhance resectability and inhibit local recurrence[19,21]. A Phase II trial published in 1993 demonstrated a significant reduction in the incidence of positive margins and lymph nodes in tumors treated with pre-operative chemoradiation[21]. The authors concluded that negative margin resections achieved in all 10 resected patients, and the low rate of nodal metastasis (10%) may be attributable to neoadjuvant treatment.

Studies of patients with more advanced disease have also proposed that neoadjuvant therapy may result in downstaging, thereby improving the likelihood of R0 resection. In 1999 White *et al*[22] performed a study of 25 patients with locally advanced pancreatic cancer treated with neoadjuvant chemoradiation at Duke University finding that only a small percent were downstaged. 22

Table 1 Comparison of radiographic differences in common definitions for borderline resectable pancreatic cancer

Effected vessel	AHPBA/SSAT/SSO/NCCN[29]	MD Anderson[28]	Alliance[26]
SMV/PV	Abutment, impingement, encasement of the SMV/PV or short segment venous occlusion	Occlusion	Tumor-vessel interface ≥ 180° of vessel wall circumference, and/or reconstructable occlusion
SMA	Abutment	Abutment	Tumor-vessel interface < 180° of vessel wall circumference
HA	Abutment or short segment encasement	Abutment or short segment encasement	Reconstructable short segment interface of any degree between tumor and vessel wall
CA	Uninvolved	Abutment	Tumor-vessel interface < 180° of vessel wall circumference

AHPBA/SSAT/SSO/NCCN: Americas Hepatopancreaticobiliary Association/Society for Surgery of the Alimentary Tract/Society of Surgical Oncology/National Comprehensive Cancer Network; SMV/PV: Superior mesenteric vein/portal vein; SMA: Superior mesenteric artery; HA: Hepatic artery; CA: Celiac artery.

of 25 patients underwent restaging after chemoradiation, six of 22 (27.3%) had a decrease in size of the primary tumor and three of the 22 (13.6%) had overall disease regression by radiographic imaging. White *et al*[23] later reported on 111 patients with PDAC, 53 with potentially resectable and 58 with locally advanced disease who underwent neoadjuvant treatment with chemoradiation followed by restaging and surgery as deemed about 11 of 58 (19%) patients with locally advanced disease underwent resection. six of fifty-eight (11%) tumors were radiographically downstaged from locally advanced to potentially resectable by neoadjuvant. Similarly, a slightly larger study at Memorial Sloan-Kettering published in 2001 reported only 3 of 87 (3.4%) patients with locally advanced disease who received neoadjuvant therapy had significant enough responses to warrant surgical exploration[24]. Together, these studies indicate that a small, but real population exists, in which neoadjuvant therapy appears to downstage pancreatic cancer. However, the lack of sensitivity of radiographic staging of pancreatic adenocarcinoma after chemoradiation indicates that radiographic tumor downstaging may not accurately reflect the benefit of neoadjuvant therapy.

Instead, margin status and histologic response may offer more reliable evidence of the efficacy of neoadjuvant therapy. In the above-mentioned studies published by White *et al*[22] in 1999, five of eight patients with either stable disease or disease regression at the time of restaging who underwent exploration were resected. One (4.5%) was resected with negative margins and negative nodes (R0). A later study by the same group reported on 103 patients with potentially resectable or locally advanced disease that underwent neoadjuvant therapy followed by re-staging computed tomography (CT). Of 49 with locally advanced tumors on restaging CT, 11 (22%), were resected, and 6 (55%) of these were resected with negative margins, suggesting that reliance on the standard CT criteria for unresectability will deprive approximately 6 of 49 or 12% of patient of the opportunity for curative (R0) resection after neoadjuvant therapy[25].

Thus, a series of clinical observations lead to the concept of borderline resectable disease. These were well-summarized by Katz *et al*[26]; (1) complete resection of the primary tumor and regional lymph nodes is mandatory for long-term survival; (2) the incidence of margin-negative resection following surgery de novo decreases with increasing involvement of the superior mesenteric vein-portal vein (SMV/PV) and superior mesenteric artery (SMA); (3) resection of the SMV/PV and hepatic artery-but not the SMA-at pancreatectomy is associated with acceptable outcomes; (4) actual tumor regression, so called, "down-staging" of locally advanced cancers is rare following the administration of conventional cytotoxic agents alone or in combination with chemoradiation therapy; and (5) chemotherapy and/or chemoradiation may be used to select patients with favorable tumor biology and physiology who may benefit from aggressive operations.

DEFINITIONS

In general, borderline resectable pancreatic cancer is neither clearly resectable nor clearly unresectable but rather implies a greater chance of incomplete resection in the setting of upfront surgery. Many groups have proposed definitions, however there is not yet a universally accepted definition of borderline resectable pancreatic cancer.

The first published definitions were by the National Comprehensive Cancer Network (NCCN) and the MD Anderson Cancer Center[27,28] (Table 1). In 2009, a consensus statement issued by The Americas Hepatopancreatobiliary Association (AHPBA)/Society for Surgery of the Alimentary Tract (SSAT)/Society of Surgical Oncology (SSO), put forth a third definition, which was later adopted by the NCCN[29]. According to the AHPBA/SSAT/SSO/NCCN definition, borderline resectable PDAC includes tumors that display; (1) venous involvement of the SMV/PV demonstrating tumor abutment, encasement, or short segment venous occlusion, but with suitable vessel proximal and distal to the area of vessel involvement, allowing for safe resection and reconstruction; (2) gastroduodenal artery encasement up to the hepatic artery and short segment encasement/direct tumor abutment of the hepatic artery with no extension to the celiac axis; or (3) tumor-SMA involvement < 180°. This differs from the definition advocated by the M. D. Anderson Group, which is largely similar to the AHPBA/SSAT/SSO/NCCN, except it excludes tumors that abut (< 180° tumor-vessel interface) or encase (≥ 180° interface) the SMV/PV, instead considering them resect-

able. More recently, Tran Cao *et al*[30] have employed a simplified radiographic classification system-Tumor-vein circumferential interface (TVI)-grouping findings as: no interface, ≤ 180° of vessel circumference, > 180° of vessel circumference, or occlusion. The TVI system was found to be predictive of the need for venous resection, histologic venous invasion, and survival

Additionally, the MD Anderson group has also described two other patient populations, termed borderline resectable "B" and "C" based on clinical, rather than anatomic criteria: those with findings that are suggestive, but not diagnostic of metastasis and patients with marginal performance status[31]. Katz groups B and C were established to recognize clinical subgroups, in addition to the well-recognized anatomic subgroup (Katz Group A), in which staging and treatment for pancreatic cancer were unclear. Many authors acknowledge these clinical definitions, however, few have utilized Katz groups in defining study populations[32-34]. Staging and treatment in clinically defined borderline resectable disease (Groups B and C) deserves attention, however, current efforts focusing on the more widely accepted anatomic definitions have tended to take precedence.

STAGING CONSIDERATIONS IN BORDERLINE RESECTABLE PANCREATIC CANCER

Preoperative evaluation

Preoperative imaging: Optimal outcomes in management of pancreatic cancer require multidisciplinary care, utilizing information from high quality imaging. CT is the most-well studied imaging modality for the evaluation of pancreatic cancer[17,29,35]. Moreover, CT is widely available and familiar to surgeons, making it an optimal imaging study for operative planning. CT should be performed using a so-called, pancreas protocol: tri-phasic contrast (non-contrast, arterial, pancreatic parenchymal, portal venous) in thin cross sectional cuts (≤ 3 mm) with multiplanar reconstructions. While CT performed in this manner has an excellent negative predictive value for unresectability, it is not as accurate at predicting resectability[35]. This is, at least in part, due to its lack of sensitivity for identifying small hepatic and peritoneal metastases.

Recently, some studies have suggested an MRI pancreas protocol may be particularly valuable due to more sensitive visualization of sub centimeter tumors/liver metastases, peritoneal carcinomatosis, and subtle signs of vascular infiltration[36,37].

The role of PET/CT in the evaluation of potentially resectable pancreatic cancer remains unclear. To date, its suggested uses include detection of metastases in high-risk patients, improved diagnostic accuracy for purposes of operative selection and assessment of response to chemoradiation[38-40]. While PET/CT may prove useful in certain circumstances, at this time, pending additional

data, its routine use cannot be recommended.

Tissue diagnosis: While histologic diagnosis is not required for patients with presumed pancreatic cancer who are going to be treated with upfront surgery, biopsy is required prior to initiation of neoadjuvant therapy in patients with borderline resectable pancreatic cancer. Fine needle aspiration (FNA) is the preferred method for obtaining a tissue diagnosis. While this can be performed percutaneously, under ultrasound (US) or CT guidance[41], endoscopic ultrasound (EUS) with FNA is favored. Numerous studies have shown that EUS-guided FNA is a safe and cost effective means of increasing diagnostic accuracy in pancreatic cancer[42-44]. Major complications are rare with approximately 2% of patients requiring post-procedure hospitalization[45]. Additionally, EUS-FNA offers decreased potential for peritoneal seeding compared to percutaneous biopsy[46].

In cases where EUS-FNA is not possible, other mechanisms for obtaining a tissue diagnosis may suffice. Intraductal biopsy or brushings may be collected *via* ERCP[47]. This method is particularly useful in borderline resectable pancreatic cancer patients with obstructive jaundice, as these patients should be stented prior to starting neoadjuvant therapy[48,49]. Stenting these patients provides symptomatic relief, reduces risk of cholangitis, prevents coagulopathy, and normalizes LFTs - a requirement in cases where abnormal liver function might result in adverse effects on the metabolism of chemotherapeutics. In the setting of neoadjuvant therapy, expandable short metal stents are preferred as they have longer patency, and therefore are associated with a lower risk of stent occlusion and resultant complication during induction therapy[50,51]. Additionally, covered stents are associated with decreased tumor ingrowth and improved patency and are therefore preferred to uncovered stents[52,53].

Role of CA 19-9: Among many tumor antigens that have been associated with pancreatic cancer, CA 19-9 is the best validated. It is a sialylated Lewis antigen and therefore is not detectable in Lewis antigen negative individuals[54]. Unfortunately, while relatively sensitive, its specificity is suboptimal as CA19-9 levels are often elevated in association with other pancreatic and hepatobiliary pathology, obstructive jaundice in particular[55]. Still, preoperative CA 19-9 has been shown to correlate with pancreatic cancer staging and therefore, resectability[56,57]. Furthermore, post-resection CA 19-9 levels prior to initiation of adjuvant chemotherapy have been shown to have independent prognostic value and can be followed to indicate response to therapy[58-60]. As such, CA 19-9 levels should typically be drawn prior to surgery, following surgery prior to adjuvant therapy and during active surveillance.

Staging laparoscopy: Though there is no absolute consensus on its use, numerous studies have demonstrated that staging laparoscopy can detect occult metastasis

even in pancreatic cancer patients who have undergone high quality cross-sectional imaging[61,62]. Detection of occult metastatic disease such as peritoneal, capsular, or serosal implants, avoids the morbidity associated with laparotomy[63]. In some institutions staging laparoscopy is routine, however others use it selectively in patients with high risk features for advanced disease such as significant weight loss, elevated CA19-9, and borderline resectable disease[56,64,65]. It is reasonable to consider laparoscopy before administering radiation therapy, as it is unlikely that local therapy would confer benefit to patients in the setting of metastatic disease.

Vascular resection: The increasing safety and feasibility of aggressive surgical resections have been central to the evolution of the concept of borderline resectable pancreatic cancer. Still, vascular resection in PD remains an area of controversy. Several studies confirming similar outcomes after PD with SMV-PV resection in comparison to PD alone were crucial in the advent of borderline resectable disease[14,15,66,67]. Even so, two recent, large database studies have called these data into question. In 2012 Castleberry et al[68] published a study using the National Surgical Quality Improvement Program database to analyze all patients undergoing PD. They found that PD with VR was associated with significantly increased morbidity and mortality. Similarly, Worni et al[69] used the National Inpatient Sample database to show comparable increases in morbidity and mortality associated with the addition of VR to PD. These studies are subject to the criticisms of any large database study. In particular, they cannot distinguish the operations performed in which vascular resection was anticipated and planned as opposed to the vascular resection performed in the setting of vascular injury when an adherent tumor is attempted to be removed. These no doubt result in much different rates of blood loss, and morbidity. Nevertheless, these studies call attention to the continued risks associated with vascular resection and are a reminder to emphasize multidisciplinary treatment and planning prior to proceeding with surgical resection in order to reduce perioperative risk in these patients[70].

Data with regard to arterial resection (AR) are even fewer. Some groups suggest similar morbidity and mortality in PD with AR in comparison to PD alone[71,72]. However, most studies indicate that AR significantly increases morbidity and mortality and therefore recommend this approach only for the purposes of obtaining an R0 resection[73]. Additionally, some suggest that AR may provide improved survival in comparison to palliation alone[74-76].

Though not unanimously employed, SMV-PV resection is more widely accepted than AR. In either case, patient selection is paramount to achieving favorable outcomes.

TREATMENT

Despite a paucity of prospective data to support a stan-

dard treatment regimen for borderline resectable pancreatic cancer, neoadjuvant therapy is currently the preferred initial approach[77-79]. Theoretical advantages to neoadjuvant treatment include early treatment of micrometastasis, improved patient selection for surgical intervention, more effective treatment delivery, as well as the potential to achieve some degree of downstaging and/or increase the likelihood of R0 resection. In addition to providing the opportunity to treat early occult disease, neoadjuvant therapy ensures that patients undergoing resection receive multimodality therapy[80]. This is an important benefit as up to 25% of patients with resectable tumors are unable to receive post-operative therapy due to post-operative complications, prolonged recovery or deconditioning[19]. Patients with borderline resectable disease often require more complex resections and it is therefore reasonable to assume delays to receipt of adjuvant therapy may be even more significant. By identifying patients with adequate performance status to complete pre-operative chemotherapy, and tumors with more favorable biology, neoadjuvant therapy selects patients most likely to benefit from resection[81]. In principle, pre-operative treatment may also enable enhanced tumor oxygenation and drug delivery compared to the post operative state, which may result in more effective radiotherapy[82].

In 2001, Mehta et al[83] described the first prospective case series of 15 patients with "marginally resectable" PDAC as indicated by CT evidence of portal vein, superior mesenteric vein, or artery involvement. Patients were treated with 5-FU and radiation followed by re-evaluation for resection. Nine of 15 patients underwent resection, all with uninvolved margins, leading the group to conclude that chemoradiation is well tolerated, and may downstage tumors, sterilize regional lymph nodes, and improve resectability in patients with "marginally resectable" pancreatic cancer.

Landry et al[84] reported the first multi-institutional prospective study in borderline resectable PDAC, a randomized phase II trial comparing neoadjuvant regimens. From 2003 to 2005, 21 patients were identified at 10 Eastern Cooperative Oncology Groups institutions. In Arm A, 10 patients, received gemcitabine based chemoradiation, in Arm B 11 patients received induction chemotherapy using gemcitabine/cisplatin/5-FU followed by chemoradiation with 5-FU. 3 patients in Arm A and 2 patients in Arm B were resected. The median survival of resected patients was 26.3 mo. All patients received adjuvant gemcitabine for 5 cycles. The trial was terminated early due to poor accrual, however it found both neoadjuvant regimens to be tolerable, with similar resectability and survival to those reported in retrospective studies.

Aside from these prospective trials, the literature in borderline resectable pancreatic cancer consists mainly of retrospective single institution studies (Table 2).

The first report from MD Ander Cancer Center was a, retrospective review of 160 patients, divided into 3 groups defined by both anatomic and non-anatomic variables[31]. Among these included 84 patients with anatomically defined borderline resectable tumors. Patients

Table 2 Largest studies in borderline resectable pancreatic cancer

Author	Year	Study type	Study size	Number with borderline resectable (definition)	Neoadjuvant	Resected	Negative margins	Median OS (mo)
Chuong *et al*[86]	2013	Single institution retrospective	73	57 (NCCN)	Majority gemcitabine based induction chemotherapy, SBRT	56%	96%	16.4
Katz *et al*[87]	2012	Single institution retrospective	129	115 (AHPBA/SSAT/SSO/ NCCN) or 72 (MDA)	Gemcitabine based chemotherapy and chemoradiation or chemoradiation alone	84% or 78%	95%[1]	33[1]
Barugola *et al*[91]	2012	Single institution retrospective	362	27 (other)	Gemcitabine based chemotherapy and chemoradiation or chemotherapy alone	NR	NR	NR
Kang *et al*[93]	2012	Single institution retrospective	202	35 (NCCN)	Gemcitabine based chemoradiation	91%	87%	26.3
Stokes *et al*[81]	2011	Single institution retrospective	170	40 (MDA)	Capecitabine-based Chemoradiation	46%	75%	23
Chun *et al*[78]	2010	Single institution retrospective	109	109 (other) 74 received neoadjuvant[2]	5-FU or gemcitabine based chemoradiation	100%	59%[2]	23[2]
McClaine *et al*[103]	2010	Single institution retrospective	29	29 (MDA+NCCN hybrid)	Gemcitabine based chemotherapy, chemoradiation or both	46%	67%	23.3
Landry *et al*[84]	2010	Randomized Phase II trial	21	21 (other)	Gemcitabine based	24%	60%	26.3
Turrini *et al*[89]	2009	Single institution retrospective	64	49 (MDA)	5-FU/cisplatin based chemoradiation	18%	100%	24
Katz *et al*[31]	2008	Single institution retrospective	160	160 (MDA)	Gemcitabine based chemotherapy, chemoradiation	41%	94%	40

[1]Results for Americas Hepatopancreaticobiliary Association/Society for Surgery of the Alimentary Tract/Society of Surgical Oncology/National Comprehensive Cancer Network (AHPBA/SSAT/SSO/NCCN) definition of borderline resectable; [2]Results for patients who received neoadjuvant treatment. NR: Not reported.

were treated with a variety of neoadjuvant regimens incorporating chemotherapy, chemoradiation, or both, prior to planned resection. Of this group, 38% underwent resection - 97% of which were R0. The median survival of all patients was 21 mo: 40 mo for resected patients and 15 mo for patients who did not undergo resection. Since this study, multiple smaller and few similarly sized retrospective reviews have reported similar findings.

Small *et al*[85] first used the NCCN definition of borderline resectable disease in a multi center, phase II trial of lesser degree, enrolling 41 patients, including 9 with borderline resectable disease. The study used neoadjuvant full dose gemcitabine plus radiation therapy, and found that treatment was well tolerated and that 33% of were able to proceed with resection. They observed a 76% one-year survival rates, and concluded that the strategy should be further explored.

Numerous other small-scale studies demonstrate the safety and efficacy of other neoadjuvant regimens. Stokes *et al*[81] performed a retrospective review of 170 cases of PDAC and identified 40 cases of borderline resectable pancreatic cancer according to the M.D. Anderson definition (A: 30; B: 5; C: 5)[31]. These patients underwent accelerated chronomodulated capecitabine-based chemoradiation using stereotactic-based radiotherapy. About 34 of 40 (85%) borderline resectable patients completed neoadjuvant therapy and were restaged, 16 (46%) of these underwent successful resection. R0 resection rate among these patients was 75%. The group concluded that accelerated chronomodulated capecitabine-based chemoradiation with stereotactic-based radiotherapy was

an efficient and well-tolerated treatment. Most recently, Chuong *et al*[86] performed a retrospective review of 73 patients who were treated with induction chemotherapy with Gemzar, Taxotere, and Xeloda and stereotactic body radiation therapy at H. Lee Moffitt Cancer Center. This included 57 patients with borderline resectable disease as designated by the NCCN definition[27]. Among 32 borderline resectable patients who underwent resection, only one patient (3.1%) had an R1 resection, while 31 patients (96.9%) had R0 resections, and median overall survival was 20 mo. It is clear that across studies, approximately one third of patients can go on to successful resection, however, small study size, inconsistent definitions of disease and a multitude of neoadjuvant strategies make it impossible to draw other definitive conclusions from these studies.

The same constraints have also made it difficult to establish anatomic guidelines for decision-making. The Fox Chase group performed a retrospective review of 109 patients with PDAC involving the PV/SMV in an effort to better delineate the degree of involvement of the PV/SMV that best defines the group of patients who would benefit from neoadjuvant therapy and resection (borderline disease)[78]. The patients were grouped according to Ishikawa classification with types II and III equating to unilateral involvement in 67 patients, while types IV and V were used to describe bilateral involvement in 42 patients. Pre-operative chemotherapy improved resection rates and overall survival in Ishikawa types II and III (unilateral involvement), but not types IV and V (bilateral involvement). R0 resection

rates in the neoadjuvant and primary resection groups were 71% and 5%, respectively ($P = 0.0001$) for types II and III, but 41% and 23%, respectively ($P = 0.25$) for types IV and V. Similarly, median overall survival rates with and without neoadjuvant were 26 and 10 mo, respectively ($P = 0.0001$) Ishikawa type IV and V patients, were 21 and 22 mo, respectively ($P = 0.48$). While this study supports the benefit of neoadjuvant therapy in patients with Ishikawa type II and III *vs* in types IV and V, increased median overall survival in patients who underwent primary resection with types IV and V (22 mo) in comparison to types II and III (10 mo) highlight the difficulty in drawing accurate conclusions due to small study size.

More recently, Katz *et al*[87] applied Response Evaluation Criteria in Solid Tumors (RECIST) criteria to determine the effect of neoadjuvant therapy on anatomic extent and size reduction in borderline resectable PDAC. They reported on 129 patients with borderline resectable tumors who underwent neoadjuvant treatment at MD Anderson. 122 of them were restaged and of these, only 15 (12%) showed partial response by RECIST criteria. Despite this, 85 (69%) underwent resection, 81 (95%) were R0. Median overall survival of those who underwent resection was 33 mo, which did not correlate with RECIST response indicating that a lack of radiographic evidence of tumor response in PDAC is of little clinical value as prognostic or predictive marker. The authors therefore suggest aggressive surgical resection in patients with adequate performance status and absence of disease progression.

Like the United States, Asia and Europe have tended toward increasingly aggressive treatment of borderline resectable pancreatic cancer. Europeans have focused on chemotherapy rather than radiation therapy, seeking improved neoadjuvant and adjuvant regimens to control systemic disease-as this is the most common cause of treatment failure[11,88-92]. Asian countries have also employed neoadjuvant strategies, but with increased emphasis on determining how it effects surgical resection[93-96]. Additionally, they have focused on defining radiographic criteria to predict surgical outcomes as well as surgical aspect that influence outcomes, such as likelihood of R0 resection, and need for vascular resection[97-100].

Need for standardization

The lack of uniformity in the definition of borderline resectable PDAC has been an obstacle to evaluating the optimal preoperative assessment, therapeutic strategy and surgical decision-making regarding this group of patients[26]. In recognition of a growing national interest in serving patients with borderline resectable PDAC, and to establish an infrastructure in which to acquire data through multi-institutional trials, The Alliance for Clinical Trials in Oncology (Alliance), in cooperation with the Southwest Oncology Group, Eastern Cooperative Oncology Group, and Radiation Therapy Oncology Group, has received support by the NCI to conduct a multi-institutional treatment trial for patients with borderline resectable PDAC (Alliance A021101). This trial was designed as a single arm pilot study with the intent to utilize a standard objective definition based on cross-sectional imaging, and to determine if a there was a sufficient patient population to conduct cooperative group trials. The study design employs a neoadjuvant design with induction chemotherapy and chemoradiation therapy, surgery and adjuvant chemotherapy[26,84].

With an aim to establish a clear, reproducible means by which to define borderline resectable PDAC by radiologic criteria, the trial has recognized any one or more of the following identifiers of borderline resectable PDAC: (1) interface exists between tumor and the SMV/portal vein measuring 180 degrees or greater of the vessel wall circumference, and/or reconstructable venous occlusion; (2) interface exists between tumor and the SMA measuring less than 180 degrees of the vessel wall circumference; (3) a reconstructable, short-segment interface of any degree exists between tumor and the common hepatic artery; and/or (4) interface exists between tumor and the celiac trunk measuring less than 180 degrees of the vessel wall circumference.

Using this definition, the trial will evaluate the survival, outcomes and toxicity rates using 4 cycles of mFOLFIRINOX (oxaliplatin 85 mg/m^2, irinotecan 180 mg/m^2, leucovorin 400 mg/m^2, 5-fluorouracil 2400 mg/m^2) followed by external beam radiation therapy (50.4 Gy) with capecitabine (825 mg/m^2). After re-staging, patients who are deemed candidates for resection proceed with surgery followed by post-operative gemcitabine.

The use of modified FOLFIRINOX (mFOLFIRINOX) as induction therapy in the Alliance Trial is based on the superior survival and response rates observed for FOLFIRINOX in metastatic pancreatic cancer in a randomized controlled trial of 342 patients with metastatic pancreas cancer. The dosing was modified in an attempt to partially circumvent the greater toxicity associated with FOLFIRINOX in comparison to gemcitabine. While FOLFIRINOX displayed improved median overall survival (11.1 mo *vs* 6.8 mo, $P < 0.001$), median progression-free survival (6.4 mo *vs* 3.3 mo, $P < 0.001$) and objective response (31.6% *vs* 9.4%, $P < 0.001$), toxicities including neutropenia, febrile neutropenia, fatigue, vomiting and diarrhea were all worse with FOLFIRINOX[101]. The Alliance Trial is therefore utilizing a modified regimen, or mFOLFIRINOX, in which the 5-FU bolus has been dropped, but all other dosing remains the same, in an effort to reduce these toxicities.

After resection, borderline resectable pancreatic caner is treated similar to any other resected PDAC. Consequently, adjuvant chemotherapy in this trial is administered according to the standard gemcitabine regimen used following resection of PDAC[102].

This benchmark trial will assess the feasibility of multi-institutional efforts to study the subset of patients regarded as having borderline resectable disease and establish a foundation for future studies in this group of
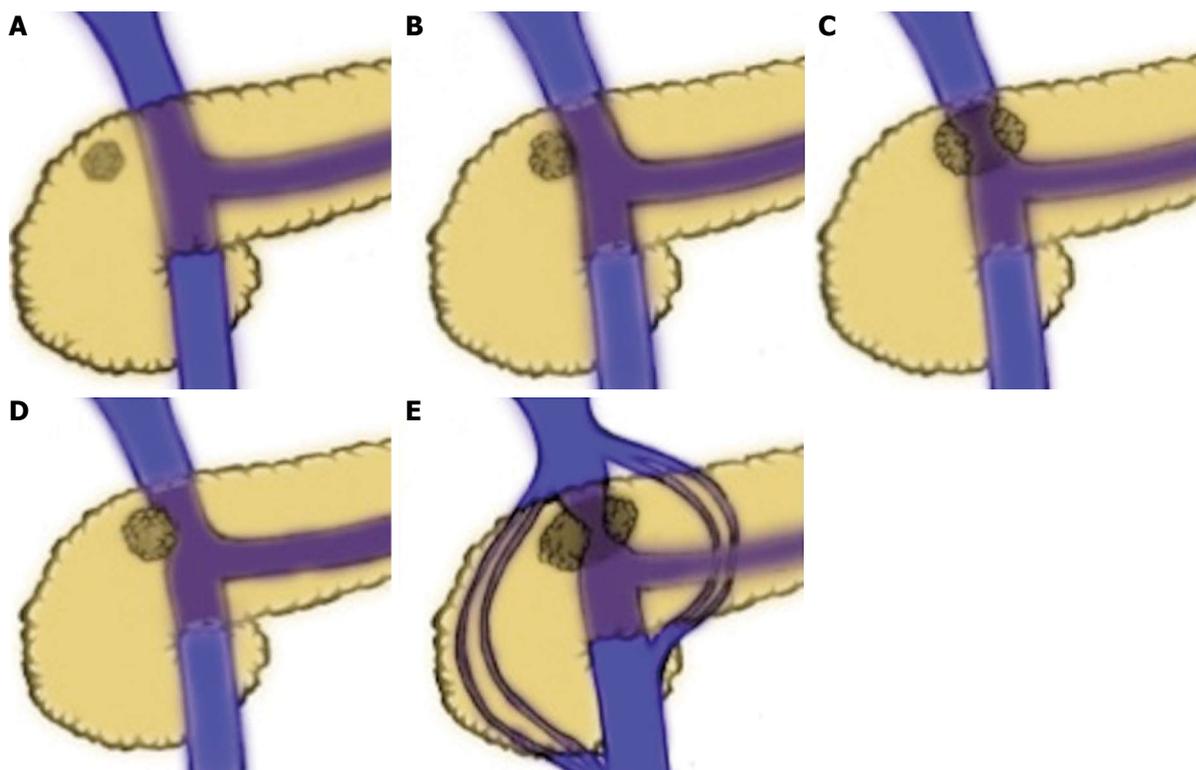
Figure 1 Ishikawa classification of portal and/or superior mesenteric vein involvement. A: Normal; B: Smooth shift without narrowing; C: Unilateral narrowing; D: Bilateral narrowing; E: Bilateral narrowing with collateral veins.

patients. While the primary endpoint of the study is, in fact, accrual, it will be of great interest to assess the activity of the neoadjuvant regimen by secondary endpoints such as the number of patients who undergo negative margin resection and overall survival. As of December 14, 2013, 14 of a targeted 20 patients had been accrued, suggesting a promising outcome for this trial.

CONCLUSION

Borderline resectable pancreatic cancer has become recognized as a clinical entity worthy of study based on a number of clinical observations that recognize a continuum between resectable and locally advanced unresectable disease. There are few prospective trials and therefore no data to support a specific neoadjuvant therapy regimen in borderline resectable PDAC. However, numerous studies suggest that patients with borderline resectable PDAC who receive neoadjuvant therapy can go on to R0 resection and enjoy outcomes similar to disease that is originally resectable[81,88,103]. Taken together the available data suggests that approximately one-third of initially borderline resectable pancreatic tumors may be proceed successful resection following receipt of neoadjuvant therapy[104]. Difficulties in achieving a consensus, objective definition, small numbers of patients and variability in therapeutic algorithms have delayed progress in establishing strong evidence-based practices for diagnosis and treatment. The Alliance trial represents a first step in establishing reproducible standards by which future trials in borderline resectable PDAC can abide.

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2014 ADVANCES IN PANCREATIC CANCER

Emerging role of the KRAS-PDK1 axis in pancreatic cancer

Riccardo Ferro, Marco Falasca

Riccardo Ferro, Marco Falasca, Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Blizard Institute, Inositide Signalling Group, United Kingdom
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Supported by Pancreatic Cancer Research Fund
Correspondence to: Marco Falasca, Professor, Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Blizard Institute, Inositide Signalling Group, 4 Newark Street, London E1 2AT,
United Kingdom. m.falasca@qmul.ac.uk
Telephone: +44-20-78828243 Fax: +44-20-78822186
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Abstract

Pancreatic cancer is a highly aggressive tumour that is very resistant to treatments and it is rarely diagnosed early because of absence of specific symptoms. Therefore, the prognosis for this disease is very poor and it has the grim supremacy in terms of unfavourable survival rates. There have been great advances in survival rates for many types of cancers over the past few decades but hardly any change for pancreatic cancer. Mutations of the Ras oncogene are the most frequent oncogenic alterations in human cancers. The frequency of *KRAS* mutations in pancreatic cancer is around 90%. Given the well-established role of KRAS in cancer it is not surprising that it is one of the most attractive targets for cancer therapy. Nevertheless, during the last thirty years all attempts to target directly KRAS protein have failed. Therefore, it is crucial to identify downstream KRAS effectors in order to develop specific drugs able to counteract activation of this pathway. Among the different signalling pathways activated by oncogenic KRAS, the phosphoinositide 3-Kinase (PI3K) pathway is emerging as one of the most critical KRAS effector. In turn, PI3K activates several parallel pathways making the identification of the precise effectors activated by KRAS/PI3K more difficult. Recent data identify 3-phosphoinositide-dependent protein kinase 1 as a key tumour-initiating event downstream KRAS interaction with PI3K in pancreatic cancer.

Core tip: Recent evidence suggests that protein kinase 1 (PDK1) is a key oncogenic driver in pancreatic cancer. Furthermore, PDK1 appears to be activated downstream the main pancreatic cancer oncogene *KRAS* that is mutated in nearly all pancreatic adenocarcinomas. This evidence suggests that PDK1 could represent a novel target in the treatment of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a deadly disease both because it is generally discovered very late but also because it is very resistant to chemotherapy and radiation therapy[1]. In addition, pancreatic cancer metastasizes very early and recent data suggest that many patients are likely to harbour metastases at the time of diagnosis[2]. The most common form of pancreatic cancer occurs in the exocrine cells of the pancreas[3]. The exocrine pancreatic tumours account for over 95% of all pancreatic cancers, and can occur anywhere in the pancreas, although most often they are found in the head of the pancreas. Pancreatic ductal adenocarcinoma (PDAC) is the most common type, repre-

senting almost 90% of all exocrine tumours.

PDACs develop from cells lining the ducts that carry the digestive juices into the main pancreatic duct and then on into the duodenum. Like other solid tumours, pancreatic cancer is the result of a multistep process. Its initiation and development involves specific genetic changes enabling growth and survival mechanisms, initiation of a marked desmoplastic reaction and finally tissue invasion and metastasis[4]. The signalling pathways regulating tumourigenesis are the result of multiple interactions between the pancreatic cells themselves, the supporting stroma and the immune system[5].

A careful molecular and pathological analysis of evolving PDAC has revealed a characteristic pattern of histologically defined precursors, named pancreatic intraepithelial neoplasia (PanIN), that has been excellently modelled by Hruban and colleagues[6]. In brief, the morphology of the tumour progresses in steps from normal ducts consisting of normal pancreatic duct cells to aberrant ducts with disorganised cell formations and differentiation grade, and finally to infiltrating cancer. These morphological changes occur along with several genetic lesions. A comprehensive genome analysis of 24 human pancreatic cancers revealed an average of 63 genetic alterations[7]. These alterations, mainly point mutations, affect distinct cellular pathways that can be classified in 12 distinct signalling pathways or processes: apoptosis, control of G1/S phase transition, Hedgehog signalling, KRAS signalling, TGF-beta signalling, Wnt/Notch signalling, DNA damage control, homophilic cell adhesion, Integrin signalling, JNK signalling, Invasion and small GTPase signalling (other than KRAS). The first six of these core pathways/processes were found to be genetically altered in all the analysed samples and the last six were altered in 16-23 of the 24 samples[7]. A recent comprehensive evaluation of the pancreatic cancer genome has revealed a multitude of additional mutated genes involved in chromatin modification and genes associated with embryonic regulation of axon guidance[1].

The progression from normal duct epithelium to infiltrating PDAC involves a series of genetic alterations in conjunction with morphological changes. Activating *KRAS* mutation and overexpression of ERBB2 occur early in the progression (PanIN-1), inactivation of the cyclin-dependent kinase inhibitor 2A at an intermediate stage (PanIN-2) and inactivation of TP53, SMAD4 and BRCA2 occur at a late stage (PanIN-3)[1,7].

Activating *KRAS* mutations are the first genetic changes that are detected in the progression from PanIN-1 to PanIN-3, even though sporadic mutation can be found in histologically normal pancreas and in lesions that show the earliest stages of histological alterations. With disease progression, the prevalence of *KRAS* mutation increases and occurs in over 90% of PDACs[1,8-10]. Understandably, KRAS-dependent pathways represent the main target in strategies attempting to counteract pancreatic cancer progression. In this review we will discuss the evidence suggesting that targeting the phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase

1 (PDK1) pathway can be a valid strategy to counteract KRAS signalling in pancreatic cancer.

KRAS

The small GTPase KRAS is frequently mutated in human cancers, with mutations occurring in nearly all tumours. Activating *KRAS* mutations involve only specific amino acids which interfere with the GTPase activity. Most mutations in pancreatic cancer change a glycine at amino acid 12 to a valine or aspartate (KRAS^{G12V} and KRAS^{G12D} respectively) and have a well-established role in the initiation and progression of PDAC[11,12]. The *KRAS* mutation result in a constitutively active protein that promotes persistent signalling to downstream effectors[13]. In turn, this hyperactivated signalling results in enhanced stimulation of proliferative pathways, thus conferring a growth advantage to the cancer cell. Several genetic studies have shown that activating *KRAS* mutations are necessary for the onset of pancreatic cancer[14]. An inducible pancreas-specific expression system was used recently to show that KRAS^{G12D} expression is also required for tumour maintenance[15]. In addition to cancer, *KRAS* mutations have also been identified in benign conditions such as chronic pancreatitis which result in increased risk of developing PDAC[16]. KRAS signals *via* a number of downstream effectors, amongst others RAF kinase, PI3K, guanine exchange factors for the small GTPases RAL (RAL-GEFs) and phospholipase Cε. In PDAC the main signalling pathways downstream of KRAS are the PI3K pathway and the mitogen-activated protein kinase (MAPK) cascade. Studies in pancreatic duct epithelial cell systems have demonstrated that the transforming potential of oncogenic *KRAS* is dependent on PI3K signalling and mutated KRAS is associated with up-regulation of survival signals including the PI3K/Akt survival pathway[17]. Knock-down of KRAS in pancreatic cancer cells demonstrated reduced activation of several proteins including Akt and ERK, indicating a key role for KRAS in regulation of the PI3K signalling pathway and the MAPK signalling cascade. Members of the MAPK network are rarely genetically modified in pancreatic cancer but this signalling pathway can be hyperactivated by constitutively active KRAS. Indeed targeting the RAF/MEK/ERK pathway in the MAPK cascade with selective drugs has shown promising effects on pancreatic cancer growth. The MAPK cascade and the PI3K pathway are both classically activated *via* Receptor Tyrosine Kinases like the epidermal growth factor receptors (EGFR). Since EGFR gene (*ERBB2*) amplification is one of the early genetic events in the development of pancreatic neoplasia these pathways can be further activated through EGFR in pancreatic cancer[18].

PI3K PATHWAY

The PI3K pathway is involved in inhibition of apoptosis and stimulation of cell proliferation and it has been estimated that at least 50% of all cancer types are related to dereg-

ulation of this signalling pathway[19]. Of the 8 mammalian PI3K isoforms gain of PIK3CA (PI3K/p110α) function by mutation is common in several human cancers[20,21]. On the other hand we have recently shown that the PI3K isoform p110γ is specifically overexpressed in PDAC[22]. Upon activation PI3Ks catalyse the phosphorylation of phosphoinoisitides promoting recruitment of downstream signalling molecules such as Akt and PDK1 to the plasma membrane which in turn induce several physiological functions such as cell growth, cell survival, cell migration, and cell cycle entry[23]. This activation is negatively regulated by the tumour suppressor phosphatase and tensin homolog (PTEN)[24]. PTEN mutations are rare in human PDAC, but loss of PTEN function has been shown to be involved in pancreatic cancer resulting in sustained PI3K activation[25]. Furthermore, animal models with KRAS[G12D] activation and PTEN deletion develop pancreatic cancer with an accelerated phenotype of acinar-to-ductal metaplasia, leading to PanIN and cancer progression[26].

Increased activation of the PI3K effector Akt was shown to be a common feature and a biological indicator of aggressiveness in PDAC[27,28]. Additionally, it has been reported that Akt is a regulator of cell plasticity in the pancreas. Indeed it has been shown that constitutively active Akt induced expansion of the ductal compartment, and also led to premalignant lesions *in vivo*[29].

PI3K signalling in the microenvironment has further been demonstrated to enhance tumour progression. Specifically, blocking PI3K/p110γ expressed by myeloid cells in the stroma significantly suppresses tumour growth and invasion[30].

KRAS/PI3K/PDK1 AXIS

It has been recently shown that PDK1 is required for anchorage-independent and xenograft growth of breast cancer cells harbouring either *PI3KCA* or *KRAS* mutations[31]. The most compelling evidence for the existence of a KRAS/PI3K/PDK1 axis derives from a recent study demonstrating that PI3K-PDK1 signalling is an essential node of non-oncogene addiction in KRAS-driven pancreatic cancer initiation and maintenance[32].

Indeed, using genetic and pharmacological approaches KRAS/PI3K/PDK1 axis has been shown to be an essential pathway for pancreatic cancer being able to induce cell plasticity, acinar-to-ductal metaplasia, intraepithelial neoplasia, and pancreatic cancer formation as well as tumour maintenance. Interestingly, the authors further showed that ablation of PDK1 specifically in the epithelial compartment of the lung using two different recombination strategies, had no significant inhibitory effect on KRAS[G12D]-induced Non-small-cell lung carcinoma (NSCLC) development and progression, supporting the conclusion that PDK1 might have a specific role downstream of KRAS in pancreatic cancer. Nevertheless, more evidence is required to conclude that PDK1 has a specific role downstream of KRAS in pancreatic cancer.

On the other hand, this demonstrates that there are substantial tissue- and context-specific differences in activation of KRAS effectors. Such differences may have important clinical implications because they could explain the diverse response to targeted therapies of different tumour types harbouring oncogenic KRAS mutations. Indeed, a recent study showed no substantial response of KRAS[G12D]-driven NSCLC toward PI3K-mTOR inhibition *in vivo*[33]. We have recently reported that the PDK1-specific inhibitor 2-*O*-benzyl-*myo*-inositol 1,3,4,5,6-pentakisphosphate (2-*O*-Bn-IP5), strongly reduced the number of surviving pancreatic cancer cells *in vitro*[34]. Our data further revealed that 2-*O*-Bn-IP5 is able to sensitise cancer cells, including pancreatic cancer cells, to the pro-apoptotic effect of anti-cancer drugs. Our data thus provide further evidence for the rationale to investigate KRAS-driven oncogenic pathways in a tissue- and context-specific manner to characterize the relevant nodes engaged in different tumour entities.

Interestingly, recent work has revealed that PDK1 directly phosphorylates the Polo-like kinase 1 (PLK1) which in turn induces MYC phosphorylation[35]. This novel PDK1-PLK1-MYC signalling regulates cancer cell growth and survival. In addition, it has been shown that MYC controls generation of self-renewing metastatic pancreatic cancer cells[36]. Indeed stable expression of activated KRAS[G12D] confers a large degree of phenotypic plasticity to cells that predisposes them to neoplastic transformation and acquisition of stem cell characteristics. Ischenko *et al*[36] demonstrated that metastatic conversion of KRAS[G12D]-expressing cells, that exhibit different degrees of differentiation and malignancy, can be reconstructed in cell culture, and that the proto-oncogene c-MYC controls the generation of self-renewing metastatic cancer cells. These results provide evidence that the conversion of precancerous to cancerous cells is determined by oncogenic RAS-induced transcription factors, primarily MYC. In addition, a cooperative mechanism between mutant *KRAS* and *PIK-3CA* has been recently shown, in part mediated by RAS/p110α binding, as inactivating point mutations within the RAS-binding domain of PIK3CA significantly ablates signalling pathways[37]. Indeed somatic cell knock-in of both KRAS[G12V] and oncogenic PIK3CA mutations in human breast epithelial cells results in cooperative activation of the PI3K and MAPK pathways in vitro, and leads to tumour formation in immunocompromised mice. Xenografts from double knock-in cells retain single copies of mutant *KRAS* and *PIK3CA*, suggesting that tumour formation does not require increased copy number of either oncogene. More importantly PDK1 seems to play a key role in this cooperativity, since PDK1-dependent activation of the downstream effector p90RSK is increased by the combined presence of mutant KRAS and PIK3CA. Finally, PDK1 has been recently found significantly overexpressed in the high-grade intraductal papillary mucinous neoplasms (IPMN) *vs* low-grade IPMN and in pancreatic and intestinal-type of IPMN *vs* gastric-type of IPMN[38]. These data suggest that PDK1 may play a role in development of IPMN invasive cancer.

MIR-375, AN ADDITIONAL LINK BETWEEN KRAS AND PDK1

MicroRNAs (miRNAs) modulate the expression levels of mRNAs and proteins and can contribute to cancer initiation and progression[39]. In addition to their intracellular function, miRNAs are released from cells and shed into the circulation. Increasing interest has been recently focused on the role of miRNAs in pancreatic cancer malignant progression[40]. It has been reported that changes in miRNAs expression patterns during progression of normal tissues to invasive pancreatic adenocarcinoma in the p48-Cre/LSL-KRAS^{G12D} mouse model mirrors the miRNAs changes observed in human pancreatic cancer tissues[41]. It was found that the expressions of miR-148a/b and miR-375 were decreased whereas the levels of miR-10, miR-21, miR-100 and miR-155 were increased in invasive carcinoma compared to normal tissues in the mouse model. Similar data have been found in KRAS oncogene transgenic rats with PDAC[42]. Recently, miR-375 has been found downregulated in different cancers including pancreatic cancer, and suppresses key cancer functions by targeting several signalling molecules such as PDK1[43]. It is worth noting that RAS can up-regulate PDK1 expression. Indeed, it has been shown that RAS drives monocytic lineage commitment in granular monocyte bipotential cells by promoting the expression of PDK1[44]. Interestingly, a recent study investigated the transcriptional regulation of miR-375 validated target PDK1[45] in pancreatic carcinoma[46]. miR-375 was observed to be downregulated in the tumour compared with non-tumour tissues from patients with pancreatic cancer[41]. As determined by a luciferase reporter assay, the ectopic expression of miR-375 was able to reduce the transcriptional activity of PDK1 and the expression of endogenous PDK1 protein levels. Functional assays showed that miR-375 was able to inhibit proliferation and promote apoptosis of pancreatic cancer cells[46]. Therefore, miRNA-375 appears to be a key regulator of PDK1, suggesting that it may have a potential therapeutic role in the treatment of pancreatic cancer. Furthermore, this evidence suggests that miR-375 may represent an additional link between KRAS and PDK1 since KRAS-induced downregulation of miR-375 results in increased PDK1 expression.

CONCLUSION

This review provides evidence for a role of the KRAS/PDK1 axis in pancreatic cancer. Given the fact that KRAS is considered an "undruggable" protein the identification of downstream targets is of value for the future development of alternative pharmacological strategies to block KRAS-dependent signalling pathways. Highly selective PDK1 inhibitors are now available and combination strategies may achieve more effective blockade of this axis. At AACR 2012, a study demonstrated that nanoparticles delivery of a novel AKT/PDK1 inhibitor inhibits pancreatic cancer tumour growth[47]. MicroRNAs may provide alternative strategies for intervention. For instance miR-375 that is downregulated in pancreatic cancer can be used as an alternative strategy to counteract the KRAS/PDK1 axis. Interestingly, miR-375 has been found downregulated in multiple types of cancer, and suppresses core hallmarks of cancer by targeting several important oncogenes such as Yes-associated protein 1 (YAP1), insulin-like growth factor 1 receptor (IGF1R) and PDK1[43]. These oncogenes might play a key role in pancreatic adenocarcinoma progression. For instance, YAP1 has been found overexpressed in pancreatic cancer tissues and might play an important role in pancreatic cancer growth[48]. More importantly, IGF1R is emerging as a novel promising new drug targets in pancreatic cancer therapy[49]. Therefore, the understanding of the role of the KRAS/PDK1 axis in pancreatic cancer might provide a number of novel therapeutic opportunities for a cancer that urgently needs immediate response to counteract its grim reality.

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2014 ADVANCES IN PANCREATIC CANCER

Role of endoscopic ultrasound in the molecular diagnosis of pancreatic cancer

Barbara Bournet, Marion Gayral, Jérôme Torrisani, Janick Selves, Pierre Cordelier, Louis Buscail

Barbara Bournet, Louis Buscail, Department of Gastroenterology, CHU Toulouse Rangueil, University of Toulouse, 31059 Toulouse, France

Barbara Bournet, Marion Gayral, Jérôme Torrisani, Janick Selves, Pierre Cordelier, Louis Buscail, INSERM UMR 1037, CHU Toulouse Rangueil, University of Toulouse, 31059 Toulouse, France

Janick Selves, Department of Pathology, CHU Toulouse Purpan, University of Toulouse, 31059 Toulouse, France

Author contributions: Bournet B, Selves J, Cordelier P and Buscail L collected and analysed the data, designed and wrote the manuscript; Gayral M and Torrisani J wrote and revised the manuscript.

Correspondence to: Louis Buscail, MD, PhD, Department of Gastroenterology, CHU Toulouse Rangueil, University of Toulouse, 1 avenue Jean Poulhès, TSA 50032, 31059 Toulouse, France. buscail.l@chu-toulouse.fr

Telephone: +33-5-61323055 Fax: +33-5-61322229

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Abstract

Pancreatic ductal adenocarcinoma remains one of the most deadly types of tumor. Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a safe, cost-effective, and accurate technique for evaluating and staging pancreatic tumors. However, EUS-FNA may be inconclusive or doubtful in up to 20% of cases. This review underlines the clinical interest of the molecular analysis of samples obtained by EUS-FNA in assessing diagnosis or prognosis of pancreatic cancer, especially in locally advanced tumors. On EUS-FNA materials DNA, mRNA and miRNA can be extracted, amplified, quantified and subjected to methylation assay. *Kras* mutation assay, improves diagnosis of pancreatic cancer. When facing to clinical and radiological presentations of pseudo-tumorous chronic pancreatitis, wild-type *Kras* is evocative of benignity. Conversely, in front of a pancreatic mass suspected of malignancy, a mutated *Kras* is highly evocative of pancreatic adenocarci-noma. This strategy can reduce false-negative diagnoses, avoids the delay of making decisions and reduces loss of surgical resectability. Similar approaches are conducted using analysis of miRNA expression as well as Mucin or markers of invasion (S100P, S100A6, PLAT or PLAU). Beyond the diagnosis approach, the prediction of response to treatment can be also investigated form biomarkers expression within EUS-FNA materials.

Core tip: This review depicts the widespread potential for the molecular analysis of samples obtained by ultrasound-guided fine needle aspiration in assessing diagnosis or prognosis of pancreatic adenocarcinoma, as well as translational studies on new markers and epigenetic alterations. Among these markers, *Kras* oncogene assay appears now the most robust for improvement of positive and differential diagnosis of pancreatic cancer. Clinical implication of miRNA, Mucins and markers of invasion is still debated. Future molecular developments may open windows towards personalized treatments after molecular characterization of a single patient.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) remains one

of the most deadly types of tumor. The 5-year survival rate after diagnosis is < 3.5%[1]. The only curative treatment is surgical resection but this surgery is possible in only 10% to 15% of cases. The remaining cases with locally advanced and/or metastatic pancreatic cancer are treated in a palliative way with chemotherapy (Gemcitabine or FOLFIRINOX) or best supportive cares[1]. This dismal prognostic is partly due to the lack of robust markers for the early diagnosis of PDAC that may jeopardize treatment efficacy in a subset of patients. Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is a rapid, safe, cost-effective, and accurate technique for evaluating and staging pancreatic tumors[2-6]. In addition, EUS-FNA is the main clinical appliance for cytological and histological material collection from locally advanced PDAC that represents 85% of pancreatic cancer patients. However, its accuracy for the diagnosis of malignancy varies widely with a sensitivity ranging from 65% to 95%, and with a mean accuracy of 85% (negative predictive value ranging from 50% to 70%). Despite the miniaturization of histological samples provided by the FNA using 22 Gauge needle, immunohistochemistry can be achieved when micro biopsies are collected, fixed and embedded in paraffin. In our experience, micro-biopsies can be thus obtained in near 80% of cases. These Immunodiagnostic can be useful to differentiate for instance PDAC from endocrine tumors. It is harder to differentiate malignant from inflammatory lesions of exocrine pancreas. Despite modern imaging techniques, difficulties persist to early diagnose PDAC and to differentiate PDAC from benign diseases such as chronic pancreatitis especially in its pseudotumoral form[2-5]. It is indeed critical to avoid unnecessary resection of benign lesions (such as focal lesions of chronic pancreatitis or autoimmune pancreatitis) or to delay the treatment of PDAC in a subset of patients. Finally EUS-FNA may be inconclusive or doubtful in up to 20% of cases[2-7]. An explanation for an inconclusive cytopathology is multiple: (1) in PDAC the presence of desmoplastic reaction often associated with poor cellularity; (2) distinguishing well-differentiated PDAC and reactive atypia is difficult to appreciate in small samples; (3) small tumors are often not easy to biopsy and performances of cytopathology are lower[7]; and (4) well vascularized tumors that have a high risk of coagulating within the FNA materials. In cases where there is an inconclusive biopsy, a doubt persists regarding the presence or not of malignancy. Some technical improvements have been developed such as contrast ultrasound, elastography, new generations of needle (pro-core biopsy needle), or transport media for samples[8-11]. However, a subset of samples remained inconclusive and accuracy of EUS-FNA is still around 80%-85%. In parallel, the improvement of molecular biology techniques including DNA and RNA amplification permits the analysis and the quantification of molecular markers in cytological samples, especially from EUS-guided FNA of pancreatic lesions[12-17]. In addition, EUS-FNA that allows sampling of biological material and molecular biology is mandatory not only for pathologists but also for scientists to discover new molecular biomarkers for this disease. This review depicts the widespread potential for the molecular analysis of samples obtained by EUS-FNA in assessing diagnosis or prognosis of PDAC, as well as translational studies on new markers and epigenetic alterations.

POTENTIAL OF MOLECULAR ANALYSIS ON EUS-FNA MATERIALS

DNA extraction
Despite using fine needles, sufficient materials can be obtained for cytology and histology. A portion of this material, collected following air or saline flushing of the needle once the core biopsies have been reclaimed for histopathology, can be used for further molecular analysis. A mean of 550 nanograms of DNA (range 100 nanograms to 1.5 mg) is obtained and DNA amplification is possible in 98 to 100 of cases[18]. For comparison, previous studies and protocols conducted on pure pancreatic juice attested for a lack of extraction/amplification in almost 13% of samples[19-21]. Thereafter, purified DNA authorizes PCR followed by Restriction Fragment Length Polymorphism or sequencing. Recently we developed an allelic discrimination assay on material sampled on EUS-FNA as well as specific Methylation-Specific PCR assay[22]. All these procedures are successful in almost 100% of the cases, in the absence of DNA pre-amplification. This is of prime importance because DNA amplification generates mutations especially when using a low amount of starting material that can eventually bias subsequent analysis. In addition, new development of large-scale sequencing allows analysis of 400 genes simultaneously with a minimal quantity of DNA of 50 ng DNA. High volume for sequencing is also offered with a mean value of 1.5 μg. That opens a window to large-scale molecular analysis from a single EUS-FNA materials and from a single patient.

RNA extraction
While material collected from pancreatic tumor or inflammatory tissue is less exposed to RNAse digestion as compared to normal pancreatic tissue, the risk of degradation is very high if one wants to analyze high-quality RNA. From a practical point of view, cytological samples should be immediately stored in transport medium (such as RNable) and frozen at -25 ℃ until use. After centrifugation, total RNA can be extracted using Micro kits (for example RNeasy from Qiagen) followed by DNAse treatment. At this crucial stage, RNA quality and quantity should be determined with specific bioanalyzer (for example Biorad Experian analyser and Agilent Technologies). RNA samples that are highly degraded (RNA 18S/28S ratio less than 1) or with a quantity lower than 5 ng should be discarded. Indeed, degraded RNA are not suitable for reverse transcription or amplification. In our experience, near 50% of FNA materials appears non available for a reliable mRNA analysis. For assay of

| Table 1 | Main studies investigating Kras mutation assay on specimens obtained by endoscopic ultrasound-guided fine-needle aspiration for the differential diagnosis between pancreatic carcinoma and pseudo-tumorous chronic pancreatitis |||

Ref.	Patient	Sensitivity (%)	Specificity (%)	Overall accuracy (%)
	PC/CP	Cytopathology alone/*Kras* + cytoP	Cytopathology alone/*Kras* + cytoP	Cytopathology alone/*Kras* + cytoP
Tada *et al*[31]	28/8	62/81	100/100	71/85
Pellisé *et al*[35]	33/24	97/97	100/100	84/98
Takahashi *et al*[34]	62/15	84/94	100/100	CytoP alone: 58
Maluf-Filho *et al*[33]	57/11	82/90	97/47	59/89
Bournet *et al*[28]	129/27	83/88	100/100	72/90
Reicher *et al*[30]1	34/16	88	94	90
Ogura *et al*[29]	307/47	87/93	100/100	89/94
Ginestà *et al*[32]	43/18	76/86	100/100	82/90
Bournet *et al*[39]2	104/72	71/90	100/99	84/94

[1]Combination of *Kras* + cytoP + FISH/Fluorescence *in situ* hybridization; [2]PC *vs* others malignant and benign pancreatic lesions. PC: Pancreatic carcinoma; CP: Chronic pancreatitis; CytoP: Cytopathology.

qPCR for 3 to 5 molecular markers an amplification is theoretically not required but if analysis on a larger panel of molecular targets is mandatory, amplification should be performed. Using 5 ng of total RNA (not degraded) is sufficient to perform RNA amplification kits (for instance Full Spectrum Kit) that permit up to 500-fold amplification with satisfactory reproducibility and reliability. In other terms, the RNA amplification from EUS-FNA material preserves the pattern of gene expression and is not influenced by the origin of the sample[23]. We had thus apply the technology of Taqmann Low Density Array to assess simultaneously the quantitative expression of 20 to 50 genes from EUS-FNA cellular materials (see below).

Micro RNA extraction

Interestingly, microRNAs are small molecules (19-25 nucleotides) of non coding RNA with high stability (less prompted to be degraded by RNase) in tissues and fluids. Moreover, they can be quantified in very low amounts of material and in highly degraded samples. Prior to microRNA analysis, tissues can be stored either frozen, or formalin-fixed and paraffin-embedded or in specific medium such as RNARetain[24]. It is important to mention that microRNA analysis of pancreatic FNA samples is possible but still in its infancy and may prove essential to help clinicians for the diagnosis of pancreatic lesions.

GENETIC MARKERS

Kras oncogene

The molecular mechanisms underlying pancreatic oncogenesis remain partially understood. However, several genetic alterations are well characterized in PDAC such as codon-12 *Kras* mutation (75% to 95%) and to a less extend *p16 (CDKN2A, INK4)*, *DPC4* and *p53* gene mutations[25,26] associated to a loss of heterozygosity of respectively 9p21, 18q and 17p. These somatic genetic alterations are also detected in pre-cancerous lesion of PDAC as intraepithelial neoplasias (PanIN) and intraductal papillary mucinous neoplasm (IPMN)[26]. Previous studies conducted by we and others on pure pancreatic

juice obtained by ERCP concluded that *Kras* mutation was found in 60% to 65 % of PDAC[19,20]. Moreover, additional *p16* and *DPC4* mutations analysis in pure pancreatic juice did not improve the sensitivity and specificity of *Kras* mutation analysis alone for diagnosis of PADC and to differentiate PDAC from CP[21]. Several research groups, including ours, have demonstrated that *KRAS* mutations can be detected in cellular materials obtained by EUS-FNA[27-37]. *Kras*-mutation analysis after EUS-FNA appears to be highly accurate at differentiating benign *vs* malignant pancreatic solid lesions[27-35].

We have conducted a multicenter prospective study to assess whether combining EUS-FNA with *KRAS*-mutation analysis could facilitate a differential diagnosis between PDAC and CP in a subgroup of patients with pseudo-tumorous forms[28]. We concluded that, when facing to clinical and radiological presentations of pseudo-tumorous CP, both pathological analyses (inflammation, fibrosis) and wild-type *Kras* are evocative of benignity. Based on the combination of cytopathological (including a second biopsy in case of negative results at the first biopsy) and *Kras* mutation analysis a medical or surgical conservative treatment can be applied. Otherwise, unnecessary pancreatic resection could be avoided. Conversely, when facing a clinical and radiological presentation of CP the presence of mutated *Kras* at EUS-FNA may justify a second biopsy and a follow up to rule out a PADC.

Whether the combination of EUS-FNA plus the *Kras*-mutation assay can improve diagnosis of malignant pancreatic tumors is still debatable. However, several studies have suggested that combining cytopathology and *Kras*-mutation analysis, improves the diagnosis of PDAC and malignancy (Table 1). This appears crucial in case of inconclusive or doubtful diagnosis at cytopathology. Inconclusive specimens were defined as the presence of coagulum with normal cells or acellular samples. Doubtful samples can be defined by the presence of atypia and/or low-grade dysplasia. Even if molecular biology cannot replace histology, the presence of a *Kras* mutation in EUS-FNA material indicates several possibilities: either immediate re-reading of the cytopathology (especially if doubtful) or a rapid indication from a sec-
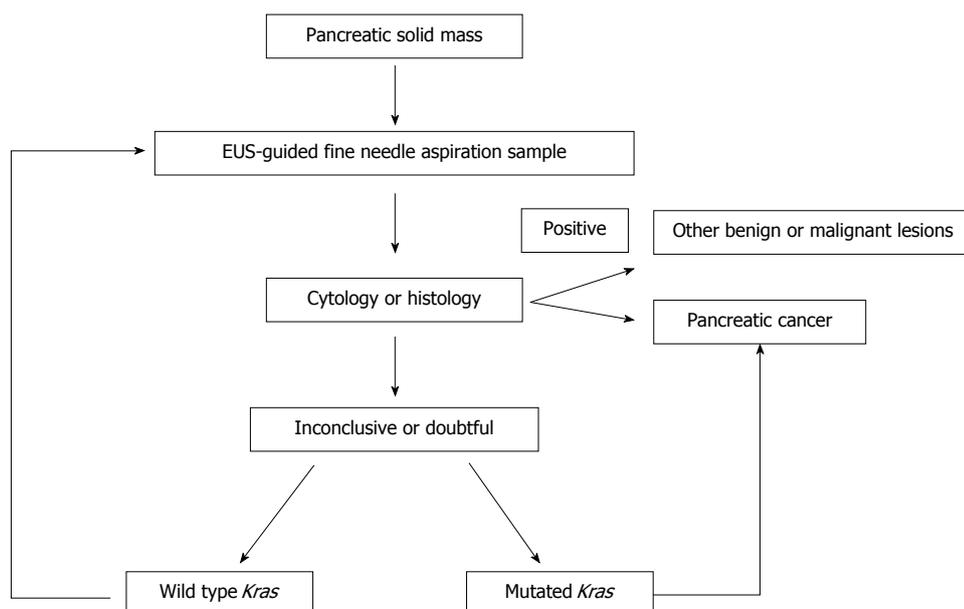
Figure 1 Integration of *Kras* mutation assay coupled to cytopathology in the algorithm of diagnosis of pancreatic cancer using endoscopic ultrasound-guided fine needle aspiration. EUS: Endoscopic ultrasound.

ond FNA, or surgery. In addition, from a clinical point of view, reducing false-negative diagnoses avoids the delay of making decisions, improves patients' treatment, and reduces loss of surgical resectability. Conversely, in cases where there is an inconclusive EUS-FNA specimen, the presence of wild-type *Kras* may be evocative of benignity. Figure 1, integrates these conclusions in a proposed algorithm that include *Kras* mutation assay in the diagnosis approach of pancreatic solid masses using EUS-guided FNA. Because *Kras* analysis is now widely available, due to its use as a predictive marker for anti-EGFR therapy in colon cancer, this diagnostic tool could also be applied to help clinicians manage solid pancreatic masses. *Kras* assay has been improved by means of Taqmann Allelic discrimination that is cheaper, faster and more selective than other previous methods[38]. We have conducted recently a prospective study that included 186 patients with a pancreatic mass (including 104 PDAC, 22 other malignant pancreatic tumors and 60 benign lesions). Cytopathology and *Kras* mutations, using TaqMan® allelic discrimination, were performed on EUS-guided FNA material. We concluded that EUS-guided FNA plus *Kras*-mutation analysis, using allelic discrimination, is accurate and improves the diagnosis of pancreatic adenocarcinoma when pathology is inconclusive or doubtful (Table 1)[39]. In addition, we also confirmed that, when facing a clinical, radiological presentation of pseudo-tumorous chronic pancreatitis (including an evocative cytopathology), identification of wild-type *Kras* can rule out malignant transformation[39]. A retrospective study that included PDAC patients but also patients with an autoimmune pancreatitis reported also that a *Kras* mutation in EUS-guided FNA material from a pancreatic mass is associated with malignancy and may help discriminate from benign conditions such

as autoimmune pancreatitis. In the study from Khalid *et al*[36] all of autoimmune pancreatitis cases had a wild type *Kras*.

Several groups, including ours, investigated whether presence or not of *Kras* mutation can influence prognosis of PDAC, especially in advanced tumors that are only investigated through EUS-FNA. Four recent studies have reported *Kras* mutations in advanced PDAC, though no correlation was found between *Kras* mutations and patient survival[40-43]. Conversely, three other published studies (one that included patients with resected PDAC, and two that included mixed populations with resected and locally advanced/metastatic PDAC) suggest that the presence of *Kras* mutations in tumor tissues have a significant adverse impact on median survival time[44-46]. Therefore, it is still difficult to conclude that the presence of *Kras* mutations influences or does not influence the prognosis of advanced PDAC. To gain further insights and to obtain a definitive conclusion, investigations on a larger cohort of similar patients (to allow strong multivariate analysis) are needed.

Others molecular alterations

Itoi *et al*[47] conducted a p53 immunohistochemical analysis in FNA biopsy specimens obtained from CP and pancreatic cancers. They reported that p53 protein overexpression was observed in 67% of the samples with pancreatic cancer, but not in samples with chronic pancreatitis, and they found that by using the combination of p53 protein overexpression and conventional histological examination, the diagnosis of pancreatic cancers improved as follows: 90% sensitivity, 91% specificity, and 92% accuracy, whereas the conventional histological EUS-FNA diagnostic test statistics for the pancreatic masses were as follows: 76% sensitivity, 91% specific-

ity, and 79% accuracy. Jahng *et al*[48] reported that the combination of p53 and cytology to detect malignancy increased the sensitivity to 51% with 100% specificity, whereas cytology alone had 41% sensitivity and 100% specificity. Salek *et al*[49] reported also that EUS-guided FNA cytology combines with screening of *Kras* mutations and allelic losses of tumor suppressor p16 and DPC4 represents a very sensitive approach particularly in cases where FNA has been inconclusive. Another group recently investigated with the same issue the quantitative analysis of MMR genes[50].

MICRORNA

MicroRNA: from basics to clinics?

MicroRNAs are small non coding RNA that functions as translation inhibitors of messenger RNA mainly following binding to 3'-untranslated region[51-53]. This mechanism is conserved from plants to humans. These molecules are tightly involved in the regulation of many physiological processes. Indeed they regulate more than 30% of mammalian gene products. In addition, microR-NAs play important roles in many diseases, including cancer, cardiovascular disease, and immune disorders. Besides their high stability in tissues and fluids, microR-NAs can repress the expression of dozens or hundreds of genes, making them an attractive therapeutic target.

MicroRNA expression is finely regulated by epigenetic modification (DNA methylation of promoters encoding for microRNA), change in DNA copy numbers, and genetic mutations[54]. For example miR-21 production is increased by *Kras* (G12D) or EGFR and decreased by TGF-b[55]. For epigenetic regulation Choi *et al*[56] described in 2012 the DNA methylation of promoter encoding for many microRNAs as a physiological process for mesenchymal stem cell differentiation. As described previously, microRNAs are very stable in tissues and fluids (urine, plasma, saliva). This is a key advantage as compare to protein or mRNA. That is why microRNAs are an emerging class of biomarkers in physiological and pathological processes, including pancreatic diseases.

MicroRNA and pancreatic cancer

microRNA expression is profoundly altered in cancer or is strongly modulated during carcinogenesis. MicroRNAs can be organized in two classes; the oncomicroRNAs which are upregulated in cancer (miR-155, miR-21)[57] and the tumor suppressor microRNAs (let-7 family) which are down regulated in cancer cells[58].

Concerning pancreatic cancer, many articles described that there is an early aberrant microRNA production in pancreatic carcinogenesis and more precisely in the development of precancerous lesions called PanIN. Indeed, the production of miR-21, miR-221, miR-222, and let-7a increased with human PanIN grade, with peak production occurring in hyperplastic PanIN-2/3 lesions[55]. Epigenetic regulation of microRNA is also described to modulate microRNA expression during pancreatic

carcinogenesis. For example, miR-148 is down regulated due to an hypermethylation of its DNA[59]. These early disturbances in the expression of microRNA persist then partly in advanced pancreatic cancer stages. In addition, many recent reports describe the alteration of microRNA expression in IPMNs, well-described non-invasive precursor lesions of pancreatic cancer[60]. Such approach may aid in diagnosis and surgical treatment decisions for patients with pancreatic cystic lesions. Taken together, microRNAs could be the ultimate biomarkers at the clinical level for the early diagnosis of pancreatic cancer and would thus allow tumor resection that is usually associated with the best prognosis.

Wang *et al*[61] were the first to report the detection of microRNA in the blood of pancreatic cancer patients. Interestingly, microRNA profiling in plasma can differentiate pancreatic cancer patients from healthy controls. They demonstrate that the plasma levels of panel of four microRNAs (miR-21, miR-210, miR-196a and miR-155) reveal a sensitivity of 64% and a specificity of 89% for pancreatic cancer. In addition, expression profiles of microRNAs may also be very informative not only to discriminate pancreatic cancer from the normal pancreas, but also for the differential diagnosis of chronic pancreatitis. This shows the interest of microRNAs as diagnostic tool in biological fluids in a non-invasive manner.

MicroRNA have been described as key players in pancreatic cancer development but above in pancreatic cancer cell chemoresistance. The mechanisms involved in drug resistance of cancer cell include alteration of drug target, altered regulation of the cell cycle and apoptosis, increased DNA damage repair and ejection of the drug from the cell by drug efflux pumps. Interestingly, microRNAs can influence the drug response by regulating all of these cellular processes[62]. MiR-21, miR-146, miR205, miR10b, miR-7 and many others microRNAs are implicated in these phenomenon. In this context, microRNAs can serve not only as a valuable therapeutic target but also as a predictive marker for a large number of diseases including pancreatic cancer. The study of microRNA expression in tumors may also lead to the identification of different molecular subtypes of pancreatic cancer that may provide insight into selection of patients likely to benefit from therapies. Nevertheless, whether this will translate into clinical applications is still highly debated.

Some microRNAs are not only predictive and diagnostic markers but also prognostic markers. Indeed, Bloomston *et al*[63] originally reported that 6 microRNAs (miR-452, miR-105, miR-127, miR-518a-2, miR-187, miR-30a-3p) are over-expressed in the patients with a longer survival (greater than 2 years). In addition, Yu *et al*[64] reported that patients with high levels of miR-200c expression present with significantly better survival rates than those with low levels of miR-200c expression.

To conclude microRNA expression signature may be informative for the diagnosis, prognosis and predictive of pancreatic cancer[65]. In other words microRNAs

Table 2 Main studies with expression of miRNA in endoscopic ultrasound-guided fine needle aspiration specimens

Ref.	miRNA	FNA	Possible clinical implication
Torrisani *et al*[58]	Let-7a	X ↓	Diagnosis
Hanoun *et al*[22]	miR-148b	X ↓	Diagnosis
Szafranska *et al*[24]			
Szafranska *et al*[24]	miR-196a	X ↑	Diagnosis
Szafranska-Schwarzbach *et al*[66]			
Szafranska *et al*[24]	miR-217	X ↓	Diagnosis
Szafranska-Schwarzbach *et al*[66]			
Du Rieu *et al*[55]	miR-21	X ↑	Diagnosis, prognosis, response to treatment
Bloomston *et al*[63]			
Preis *et al*[67]	miR-10b	X ↑	Prognosis, response to treatment

X ↑: Up regulated; X ↓: Down regulated. FNA: Fine needle aspiration.

appear to be reliable biomarkers to assist clinicians in all stages of care for patients with pancreatic cancer. Thus, microRNAs are expected in the future to prove specific and/or sensitive as a long-awaited screening tool for pancreatic cancer.

MicroRNA detection EUS-FNA

Nowadays, the vast majority of pancreatic cancer patients have metastatic and/or locally advanced diseases at the time of diagnosis; in other words, these patients are not eligible for curative resection which explains the limited access to pancreatic tissue specimens. As explain before, endoscopic ultrasound-guided fine needle aspiration-biopsy is the most widely used approach for cytological and histological material sampling in this situation. Szafranska *et al*[24] revealed that microRNA and more precisely, miR-196a and miR-217 expression analyses from FNA material can discriminate pancreatic cancer from benign lesion with a high sensitivity (90%) and specificity (100%). These results paved the way to the first development of a molecular test using microRNA for the differential diagnosis of pancreatic cancer[66].

Preis's[67] group has demonstrated that miR-10b and miR-21, two well-characterized onko microRNAs, are over expressed in the FNA material from pancreatic cancer patients. MiR-10b is now classified as a novel and powerful diagnostic biomarker for pancreatic cancer. In addition, reduced expression of miR-10b is associated with improved response to multimodality neoadjuvant therapy, surgical resection, time to metastasis, and increased survival. Thus, miR-10b may serve as a novel diagnostic and prognostic biomarker in PDAC and as a tool for predicting response to therapy.

Then, we recently demonstrated that let-7a tumor suppressor microRNA expression is repressed[58] in FNA material of pancreatic cancer and that the measurement of hypermethylation of microRNA miT-148a encoding DNA region is potentially useful to differentiate pancreatic cancer and pseudo-tumor forms of chronic pancreatitis[22].

To conclude, microRNAs are very promising emerging molecular markers in pancreatic cancer that can be quantified in EUS-FNA specimens. Table 2 resumes clinical applications of microRNAs in FNA material. However, forthcoming prospective studies are needed to ask whether microRNAs may translate into reliable biomarkers for pancreatic cancer management.

MUCINS AND MARKERS OF INVASION

Expression of mucins

Mucine (MUC) are the main components of mucus. They are synthesized and secreted by specialized cells of the epithelium and in some case, by non mucin-secreting cells. MUC are membrane-tethered high molecular weight glycoprotein, and frequently overexpressed in PDAC. Mucins have been implicated in tumorigenicity, invasiveness, metastasis and drug resistance through their characteristic *O*-linked and *N*-linked oligosaccharides, extended structures and unique domains. MUC are classified into three categories, membrane associated mucins with MUC1, MUC3 or MUC4, gel-forming mucins with MUC 2 or MUC5AC and soluble mucin with MUC7. MUC are expressed in normal pancreatic tissue, PDAC or precusors lesions as IPMN or PanIN[68]. The MUC expression profile has a hight value for the diagnosis of PDAC (especially MUC1) and several studies implicated these markers in the prognosis and outcome of patient. From samples obtained under EUS FNA, MUC can be detected using immunohistochemistry[69,70]. By this way, Nagata *et al*[68] investigated the expression of MUC in various pancreatic tissues. MUC1 and MUC6 are expressed in normal pancreatic tissues while MUC 2 and MUC 5AC are never expressed. The expression profile of MUC in IPMN is different between the different subtypes of IPMN. IPMN of intestinal type display a high expression of MUC2 and MUC5 AC while IPMN of pancreaticobiliary type has a low expression of MUC2 and a high expression of MUC 5AC. In PDAC tissues, MUC1 has a high expression but no expression of MUC2. Wang *et al*[71], after immunohistochemistry on EUS-FNA samples demonstrated the expression of MUC1, MUC2 and MUC5AC in PDAC and in benign pancreatic disease samples. They investigated the diagnosis value of cytology analysis alone *vs* combination of cytology together with the presence of MUC1 or MUC 5AC. They concluded that the combination of cytology and MUC1+ or MUC5AC+ provide higher sensibility and accuracy for the diagnosis of PDAC (Table 3).

Expression of factors implicated in tumor invasion

The identification of others biomarkers has been proposed from samples of pancreatic tissue obtained by EUS-FNA. Indeed the quality and the amount of cellular pancreatic samples obtained by EUS-FNA allow immunohistochemistry thanks to cellblocks but also the extraction of RNA to perform Real Time Reverse Transcription Polymerase Chain Reaction.

We previously performed an expression study using cDNA macro array of pancreatic cancer cells and PDAC

Table 3 Main studies investigated expression of Mucin and molecular markers for the diagnosis of pancreatic cancer using endoscopic ultrasound-guided fine needle aspiration materials					
Molecular markers	Methods for analysis	Sensitivity (%)	Specificity (%)	Accuracy (%)	Ref.
MUC1+/MUC2-/MUC5AC+	IHC	70	100	75	70
CytoP + MUC5AC	IHC	90	93	91	71
CytoP + MUC1		85	100	89	
MSLN, UPAR	qRT-PCR	100	94	-	77
S100P	IHC	90	90	87	78
MSLN		62	74	66	
S100P + KRT7	qRT-PCR	80	77	-	23
cytoP alone	qRT-PCR	68/88	100/90	75/89	73
cytoP alone/cytoP + S100A6					

CytoP: Cytopathology; IHC: Immunohistochemistry; qRT PCR: Quantitative reverse transcription polymerase chain reaction; MUC: Mucin; MSLN: Mesothelin; UPAR: Urokinase plasminogen activator receptor.

tissues. Following this DNA chip study, RT-QPCR validated the increased expression of LCN2 (lipocalin 2) and for the first time PLAT (tissue-type plasminogen activator or tPA) in PDAC as compared with normal pancreas. Following, PLAT and LCN2 transcripts obtained through EUS-guided FNA from patients with PDAC showed a significant increased expression levels in comparison with those found in normal tissues, indicating that a sufficient amount of high quality RNA can be obtained with this technique[72].

Subsequently we conducted a prospective multicenter study using dedicated TaqMan Low Density Array technology on EUS-FNA materials[23]. We quantified candidate gene expression in biopsies sampled from 44 locally advanced and/or metastatic pancreatic carcinoma and from 17 pseudotumoural chronic pancreatitis. We found that eight genes (*S100P, PLAT/Plasminogen Activator Tissue, PLAU/PLasmine Activator Urokinase, MSLN/Mesothelin, Matrix MetalloProteins 7 and 11, KRT7 and 17/Keratin*) were significantly over expressed in pancreatic cancer samples when compared to pseudotumoural chronic pancreatitis. The area under receiver operating curve establishes the clinical validity of the potential diagnostic markers identified in this study (values ranging from 0.69 to 0.76). In addition, combination of S100P (calcium binding protein P) and KRT7 gave better diagnosis performances (Table 3). We demonstrate that molecular studies on EUS-guided FNA material are feasible for the identification and quantification of markers in PDAC patients diagnosed with non-resectable tumours. Using low-density array, we isolated a molecular signature of advanced pancreatic carcinoma including mostly cancer invasion-related genes[23]. Zihao *et al*[73] demonstrated the interest of S100A6 for the diagnosis of pancreatic cancer. The material used RNA extracted for quantification of gene expression by RT PCR and S100A6 immunohistochemistry to validate the expression. Deng *et al*[74] demonstrated also a nuclear or cytoplasmic staining for S100P and it was specific for pancreatic cancer with 100% diagnostic sensibility. Kosarac *et al*[75] obtained similar results. These different biomarkers as calcium binding proteins such as S100P (that are associated to metastase and poor prognosis) may contributed in positive diagnosis of

pancreatic cancer but also in differential diagnostic with benign pancreatic disease[76-78].

MARKERS FOR THE TREATMENT EFFICACY

Gemcitabine is transported into cells predominantly by human equilibrative nucleoside transporters[79]. A deficiency of activity of one of them, hENT1, conferred high-level resistance to the toxicity of gemcitabine[80-83], and patients with PDAC that have detectable hENT1 or high *hENT1* gene expression have significantly prolonged survival after gemcitabine chemotherapy when given as adjuvant treatment after resection[84,85]. After transport, Gemcitabine must be sequentially converted into mono- di- or triphosphorylated forms to exert its full cytotoxic activity. In this cascade, the first two steps of phosphorylation are rate-limiting. In mammalian cells, gemcitabine conversion into gemcitabine monophosphate is performed by the deoxycytidine kinase (DCK)[80-81]. Then, the Uridylate monophosphate kinase yields gemcitabine diphosphate[81]. Gemcitabine derivatives exhibit antitumor activity either by interfering with intracellular nucleotide pools, or through direct inhibition of DNA synthesis. Resistance to gemcitabine has been reported to involve a deficiency in DCK enzyme, a decrease in nucleoside transport and an overexpression of ribonucleotide reductase. After cellular entry, gemcitabine must be phosphorylated by deoxycytidine kinase (dCK), which is a rate limiting step. We previously demonstrated that down-regulation of dCK specifically enhanced acquired resistance to gemcitabine in pancreatic cancer cells, whereas transfection of wild-type dCK restored sensitivity to the drug[83]. Conversely, active metabolites of gemcitabine are reduced by 5'-nucleotidase, and gemcitabine itself is inactivated by cytidine deaminase (CDA). High levels of these catabolic enzymes are associated with resistance to the drug. Ribonucleic Reductase (RR) is a dimeric enzyme composed of regulatory subunit M1 and catalytic subunit M2. PDAC patients with high levels of RRM1 expression had poor survival rates after gemcitabine treatment. Moreover, RRM2 gene silencing

by RNA interference is an effective therapeutic adjunct to gemcitabine treatment. These data suggest that the genes encoding proteins involved in the transport and metabolism of gemcitabine and in the metabolism of targets can be potential candidates to predict sensitivity to gemcitabine.

Fujita *et al*[84] investigated 70 patients with PDAC. Of the 70 patients, 40 received gemcitabine-based adjuvant chemotherapy. They measured hENT1, dCK, CDA, RRM1, and RRM2 mRNA levels by quantitative real-time reverse transcription-polymerase chain reaction in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) cytological specimens. High dCK, low RRM2 groups had a significantly longer disease-free survival in the gemcitabine-treated group[85]. Itoi *et al*[86] assessed 35 PDAC biopsy specimens for RRM2 expression levels. In the low RRM2 expression group, a complete response was observed in one patient, and a partial response was observed in eight patients. In contrast, in the high RRM2 expression group, complete response was not observed. In the work from Ashida *et al*[87] mRNAs were extracted from 35 unresectable pancreatic adenocarcinoma tissues obtained by EUS-FNA before GEM-treatment. Among these GEM sensitivity-related genes, dCK alone showed a significant correlation with Gemcitabine efficacy. Among all molecules that are crucial for Gemcitabine intracellular transport and metabolism, hENT1, dCK and RRM2 appear important. If their expression has been studied at the mRNA levels on EUS-FNA, immunohistochemistry on these materials remains to be validated.

CONCLUSION

Both clinician and scientist take benefit from cellular and tissue material sampled under EUS-FNA in PDAC patients. The progress of molecular biology authorizes now extraction of DNA, mRNA and miRNA. After amplification identification of genetic abnormalities and quantification of biomarkers for improvement of diagnosis, prognosis and hopefully treatment together with a better knowledge of pancreatic carcinogenesis especially in locally advanced pancreatic adenocarcinoma. Among these markers, *Kras* oncogene assay appears now the most robust for improvement of positive and differential diagnosis of pancreatic cancer especially when FNA are inconclusive or doubtful. Clinical implication or miRNA, Mucins and markers of invasion is still debated. Future molecular developments may open windows towards personalized treatments after molecular characterization of a single patient.

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2014 ADVANCES IN PANCREATIC CANCER

Translational research in pancreatic ductal adenocarcinoma: Current evidence and future concepts

Stephan Kruger, Michael Haas, Steffen Ormanns, Sibylle Bächmann, Jens T Siveke, Thomas Kirchner, Volker Heinemann, Stefan Boeck

Stephan Kruger, Michael Haas, Sibylle Bächmann, Volker Heinemann, Stefan Boeck, Department of Internal Medicine Ⅲ and Comprehensive Cancer Center, Klinikum Grosshadern, Ludwig-Maximilians-University of Munich, D-81377 Munich, Germany

Steffen Ormanns, Sibylle Bächmann, Thomas Kirchner, Department of Pathology, Ludwig-Maximilians-University of Munich, 80377 Munich, Germany

Jens T Siveke, Department of Internal Medicine Ⅱ, Klinikum Rechts der Isar, Technical University of Munich, 81675 Munich, Germany

Author contributions: Kruger S and Boeck S wrote the manuscript; Haas M, Ormanns S, Bächmann S, Siveke JT, Kirchner T and Heinemann V critically reviewed and revised the manuscript; all authors read and approved the final version of the manuscript.

Correspondence to: Dr. Stefan Boeck, Department of Internal Medicine Ⅲ and Comprehensive Cancer Center, Klinikum Grosshadern, Ludwig-Maximilians-University of Munich, Marchioninistr. 15, D-81377 Munich, Germany. stefan.boeck@med.uni-muenchen.de

Telephone: +49-89-70952208 Fax: +49-89-70955256

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is one of the major causes for cancer death worldwide. Treatment of metastatic disease remains challenging as only certain patients benefit from advances made with the intensified chemotherapy regimen folinic acid, irinotecan and oxaliplatin, the epidermal growth factor receptor inhibitor erlotinib or the recently FDA-approved nab-paclitaxel. Up to date, no established approach for prediction of treatment response or specific treatment allocation exists. Translational research was able to identify a number of potential biomarkers that might help to improve the dismal prognosis of PDA by facilitating upfront treatment allocation. This topic highlight is focused on current evidence on potential biomarkers for tumor biology, prognosis and prediction of treatment efficacy.

Core tip: Recent advances in the treatment of pancreatic ductal adenocarcinoma (PDA) have been made using the intensified chemotherapy regimen folinic acid, irinotecan and oxaliplatin, the recently FDA-approved nab-paclitaxel and the epidermal growth factor receptor-inhibitor erlotinib. Yet overall prognosis of PDA remains poor. To further improve outcome of PDA, innovative strategies are needed to identify patient subgroups that benefit most from specific regimens. This topic highlight focuses on potential biomarkers to identify patients that benefit from treatment with erlotinib (*e.g.* KRAS, AKT, ERK, p53), gemcitabine (hENT1, RRM1, dCK), nab-paclitaxel (SPARC) or angiogenesis inhibitors. Additional biomarkers of tumor biology (like SMAD4 and CXCR4) and future concepts of translational research in PDA are also discussed.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) constitutes

the fifth leading cause of cancer death accounting for approximately 227000 annual deaths worldwide[1-4]. It is only curable by surgical resection which is feasible in about 15%-20% of all patients[4]. Non-resectable patients usually receive palliative chemotherapy with gemcitabine-based combinations[5]. However, these combinations often fail to offer long-term disease control resulting in a poor five-year survival rate of about 4%[4]. Advances in specific patient populations have been made using the tyrosine kinase inhibitor erlotinib and an intensive treatment regimen consisting of 5-fluorouracil (5-FU), folinic acid, irinotecan and oxaliplatin (FOLFIRINOX). While effects on the overall population are minimal for erlotinib, intensive chemotherapy with FOLFIRINOX is tolerated by certain patients only[6,7]. Treatment options proven to be beneficial in other cancer entities like the vascular endothelial growth factor (VEGF) inhibitor bevacizumab have failed to improve survival in an unselected PDA population[8]. Preclinical data propose that innovative agents like the recently FDA-approved albumin bound nab-paclitaxel might be dependent on expression of specific proteins, suggesting predefined patient subgroups as major beneficiaries[9]. Hence new biomarkers are urgently needed for treatment allocation and identification of patient subgroups that might benefit from alternative treatment strategies[10]. This topic highlight summarizes and assesses current evidence from translational studies on biomarkers for tumor biology, prognosis and prediction of treatment response in PDA.

Biomarkers for tumor biology and prognosis

SMAD4: SMAD4/DPC4 is a protein involved in intracellular transforming growth factor-b1 signaling[11]. In PDA differing functions as biomarker have been ascribed to SMAD4. Based on findings in rapid autopsies performed on patients previously diagnosed with stage I to IV PDA, Iacobuzio-Donahue *et al*[12] suggested SMAD4 as a biomarker for metastatic pattern of PDA. They determined presence or absence of intact SMAD4 using immunochemistry in PDA of 65 patients. Abnormal immunostaining of SMAD4 was found in 41 patients (63%). Absence of intact SMAD4 was significantly more frequent in metastatic disease (78%) and significantly reduced in locally destructive disease (22%) (*P* = 0.007). Oshima *et al*[13] screened 106 patients with PDA who had undergone surgical resection for mutations in different genes including the *SMAD4* gene. Abnormal immunolabeling for SMAD4 was detected in 64 patients (60%). Using univariate analysis, a significant correlation between tumor size (*P* = 0.006), lymphatic invasion (*P* = 0.033), lymph node metastasis (*P* = 0.006) and abnormal immunostaining for SMAD4 was found. Overall survival for patients with intact *vs* mutant SMAD4 was 30.1 mo *vs* 18.3 mo respectively (*P* < 0.001). Within a multivariate analysis mutant SMAD4 was found to be a significant and independent poor prognostic factor for both disease free and overall survival. In a different study Bachet and co-workers examined tumor samples of 471 patients with resected PDA and assessed SMAD4 status by tissue microarray analyses. Patients with mutant

SMAD4 significantly benefited from adjuvant chemotherapy (hazard ratio for death compared to untreated patients = 0.59; 95%CI: 0.42-0.82; *P* = 0.002), whereas no significant beneficial effect of adjuvant treatment was witnessed for SMAD4 wild-type status (hazard ratio for death = 0.85; 95%CI: 0.49-1.46; *P* = 0.552). While disputing a correlation between metastatic pattern and SMAD4 status, the authors conclude that SMAD4 might also predict adjuvant treatment response[14]. Using multivariate analysis Winter *et al*[15] recently examined the correlation between SMAD4 status and different clinical criteria in 127 patients with resected PDA. In harsh contrast to earlier findings they found neither a correlation between SMAD4 and metastatic pattern nor a correlation between SMAD4 and overall survival (Table 1).

CXCR4: Chemokines are small cytokines capable of inducing chemotaxis. They exert their effects *via* specific chemokine receptors found on various cell types including immune and tumor cells. The chemokine receptor CXCR4 has been described to be widely expressed in different cancer types[16]. *Via* interaction with its ligand, the chemokine CXCL12 (SDF-1α) is believed to promote tumor growth, angiogenesis and tumor dissemination[17]. In PDA two retrospective studies concluded CXCR4 to be a significant and independent poor prognostic factor for overall survival while a third study found no significant correlation between overall survival and CXCR4[14,18,19]. The CXCR4 ligand CXCL12 has recently been reported to be a predictive marker for treatment response to bevacizumab as discussed below[20]. Additional research is clearly necessary to identify the potential of CXCR4 and its ligand in predicting treatment response and prognosis in PDA.

Predictive biomarkers of the epidermal growth factor receptor pathway

The oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib modestly improves survival in an unselected patient population with metastatic PDA. However, a significant survival benefit from erlotinib treatment is observed for patients developing skin rash[5]. Erlotinib exerts its effects by inhibiting intracellular receptor transphosphorylation of the ErbB1/HER1 receptor[21]. Translational studies therefore aimed to identify EGFR polymorphisms and gene amplifications predictive for erlotinib treatment. Despite of promising findings in pre-clinical and early clinical studies, translational subgroup analyses from prospective clinical trials failed to reveal a significant correlation between genetic EGFR alterations or overexpression and treatment response to erlotinib up to now[22]. Recent investigational approaches on identifying predictive EGFR pathway biomarkers have therefore focused on downstream EGFR signaling such as the PI3K-AKT-PTEN network or the RAS-RAF-MAPK-MEK-ERK cascade.

KRAS: Mutations in members of the *RAS* gene family such as v-Ki-ras2 Kirsten rat sarcoma viral oncogene ho-

Table 1 Summary of current evidence on selected biomarkers in pancreatic ductal adenocarcinoma discussed in this topic highlight

Ref.	Biomarker	Prognostic	Predictive (for)	Dissemination pattern
Iacobuzio-Donahue *et al*[12]	SMAD4	N/A	N/A	+
Oshima *et al*[13]		+	N/A	+
Bachet *et al*[14]		-	+ (adjuvant chemotherapy)	-
Winter *et al*[15]		-	N/A	-
Bachet *et al*[14]	CXCR4	+	-	+
Maréchal *et al*[18]		+	N/A	+
Gebauer *et al*[19]		-	N/A	-
Lee *et al*[27]	KRAS	+	N/A	N/A
Shin *et al*[28]		+	N/A	N/A
Ogura *et al*[29]		+	N/A	N/A
Boeck *et al*[22,31]		+	? (erlotinib)	N/A
Kim *et al*[24]		-	+ (erlotinib)	N/A
da Cunha Santos *et al*[32]		-	-	N/A
Oliveira-Cunha *et al*[33]		-	N/A	N/A
Farrell *et al*[48]	hENT1	-	+ (adjuvant gemcitabine)	N/A
Morinaga *et al*[49]		-	+ (adjuvant gemcitabine)	N/A
Maréchal *et al*[50]		-	+ (adjuvant gemcitabine)	N/A
Greenhalf *et al*[52]		-	+ (adjuvant gemcitabine)	N/A
Jordheim *et al*[51]		-	+ (review on 18 studies on adjuvant gemcitabine)	N/A
Poplin *et al*[53]		-	- (gemcitabine in metastatic PDA)	N/A

(+): Results suggest that the respective molecule might serve as a biomarker; (-): No evidence found that the respective molecule might serve as a biomarker; (?): Unclear whether the respective molecule could serve as a biomarker. N/A: No data available; in cited study; PDA: Pancreatic ductal adenocarcinoma.

molog (KRAS) are frequently observed in different types of human cancers[23]. The highest frequency of mutant KRAS can be found in PDA. It has been reported that mutant KRAS is present in up to 90% of all PDA[10,24]. Single point mutations in codon 12, 13, 59 or 61 of exon 2 and exon 3 of the KRAS oncogene impair intrinsic GTPase activity of KRAS and lead to a permanent active KRAS signaling pathway, resulting in sustained proliferation and survival of cells[23]. KRAS has been described as a predictive biomarker for treatment success by inhibitors of the EGFR pathway such as the small-molecule drug erlotinib or the monoclonal antibodies cetuximab and panitumumab in metastatic non-small cell lung and colorectal cancer[25,26]. So far, the value of KRAS as biomarker in PDA has not been clearly established. In a retrospective study performed by Lee *et al*[27], KRAS status of 66 patients with metastatic ($n = 61$) or locally advanced ($n = 5$) PDA was analyzed. The majority of patients ($n = 64$) had received first-line chemotherapy with gemcitabine alone or gemcitabine in combination with capecitabine, uracil/tegafur (UFT) or cisplatin. In a total of 42 patients (64%) KRAS mutations were found (codon 12: $n = 41$, codon 63: $n = 1$). Comparison between patients with a mutation in codon 12 of the KRAS oncogene and wild-type KRAS showed a significant reduced overall survival for patients with mutant KRAS (9.1 mo *vs* 13.4 mo, $P = 0.03$). In conclusion Lee *et al*[27] suggested that KRAS might be of value as a prognostic biomarker in PDA. In the largest retrospective study conducted on KRAS as a biomarker in PDA so far, Shin *et al*[28] analyzed KRAS status of 234 resected PDA patients by polymerase chain reaction. Mutant KRAS was present in 126 patients (55%). Using multivariate analysis mutant KRAS was found to be significantly correlated to poor prognosis. In a different study by Ogura *et al*[29] similar findings were reported. Of

note neither Shin *et al*[28] nor Ogura *et al*[29] commented on applied chemotherapy regimens in the study population, making it impossible to distinct between a predictive and merely prognostic correlation. The AIO-PK0104 study was a large, multicenter phase III trial in advanced PDA conducted by the German AIO study group[30]. In a post-hoc analysis of AIO-PK0104 the wild-type KRAS status was found to be significantly correlated with an improved overall survival (hazard ratio for death for wild-type compared to mutant KRAS = 1.68; $P = 0.005$). Owing to the study design (all patients received erlotinib plus either capecitabine or gemcitabine as first-line chemotherapy) it was impossible to directly distinguish between a prognostic and predictive correlation[22]. However, within an exploratory analysis no significant correlation of KRAS status with objective response to erlotinib-containing first-line therapy was found, indicating that KRAS may not serve as a predictive but rather as a prognostic biomarker for overall survival in PDA[31]. Contrary to these observations, Kim *et al*[24] screened tumor samples of 136 patients with metastatic ($n = 112$) or locally advanced ($n = 24$) PDA, who had received first-line therapy with gemcitabine alone ($n = 22$) or a combination of gemcitabine with either erlotinib ($n = 70$), capecitabine ($n = 31$) or UFT ($n = 13$). In 71 patients (52%) mutations in codon 12 ($n = 70$) or codon 61 ($n = 1$) of the KRAS oncogene were found. Post-hoc analysis showed a significant difference in overall survival between patients with wild-type and mutant KRAS status treated with erlotinib and gemcitabine (9.7 mo *vs* 5.2 mo, $P = 0.002$) whereas no difference in survival was observed in patients treated with regimens without erlotinib (7.0 mo *vs* 7.0 mo, $P = 0.121$). The authors from this Asian study therefore concluded that KRAS might be a predictive but not a prognostic biomarker. In clear contrast to this conclusion

are the findings of da Cunha Santos *et al*[32], who analyzed tumor samples of 117 patients from the erlotinib pivotal trial PA.3. Mutant KRAS was present in 92 patients (79%). Comparison of overall survival showed a non-significant survival benefit for wild-type KRAS patients treated with erlotinib plus gemcitabine *vs* patients treated with gemcitabine plus placebo (6.1 mo *vs* 4.5 mo, $P = 0.34$) while patients in the wild-type KRAS arm showed a trend towards reduced overall survival under anti-EGFR therapy (6.0 mo *vs* 7.4 mo, $P = 0.78$). In a study conducted by Oliveira-Cunha and co-workers the correlation between KRAS status and overall survival in 100 patients with resected pancreatic and periampullary cancer was analyzed. The investigators reported a non-significant shorter overall survival for mutant KRAS patients (22.8 mo *vs* 28.1 mo, $P = 0.88$) and concluded that there is no correlation between KRAS and overall survival[33]. Noteworthy limitations of mentioned studies are retrospective design, lack of data on systemic therapy and vague definition of the cancer subtype investigated (*e.g.* periampullary cancer *vs* PDA). Additional research using prospective biomarker studies with clearly defined patient populations is crucial to clarify the possible use of KRAS as a prognostic or (even more important) as a predictive biomarker for treatment response to erlotinib.

ERK: EGFR signaling through KRAS is dependent on a complex interplay of intracellular proteins like the extracellular signal-regulated-/mitogen-activated protein kinase (ERK/MAPK). Because of its location downstream the RAS-RAF-MEK cascade, ERK might be useful in predicting success of anti-EGFR treatment[34]. Additionally some previous studies in PDA suggested that high ERK expression might be a poor prognostic factor while other studies found no correlation between ERK expression and survival[35-37]. The AIO-PK0104 investigators also examined the correlation between ERK expression and overall survival. Archival tumor tissue samples of 153 patients with advanced PDA who had received an erlotinib-based 1st-line regimen were analyzed using a grading system of cytoplasmic and nuclear phospho(p-)ERK expression ranging from 0 (no expression) to 12 (high expression). A significant increase in the hazard ratio for death by a factor of 1.06 for each pERK score level (0 to 12) was observed (HR = 1.06; 95%CI: 1.0-1.12; $P = 0.05$). As for KRAS (see above) it was not possible to definitely determine whether this correlation is solely prognostic or predictive for erlotinib efficacy due to the design of the trial[38].

AKT: Besides activation of KRAS, dimerization of EGFR activates phosphoinositol-3-kinase (PI3K), resulting in activation of the serine/threonine-specific protein kinase AKT. The active form of AKT, phosphorylated AKT (pAKT) is an important mediator of cell survival and protein synthesis[34]. Its activity is negatively regulated by the tumor suppressor protein phosphatase and tensin homolog (PTEN). A deregulated AKT/PTEN pathway leads to

resistance of cancer cell lines against anti-EGFR treatment *in vitro*[39]. Additionally, a correlation between expression of AKT and overall survival has been described in previous small PDA studies[35,36]. Within AIO-PK0104 tumor samples of 35 patients were categorized based on their pAKT expression level: no difference in progression free or overall survival between PDA patients expressing low or high levels of pAKT was observed[38].

p53: Mutations in the tumor suppressor gene TP53 are an important step in the oncogenesis of most human cancer types. Including PDA, approximately 80% of all malignant tumors embody mutated TP53. Its transcriptional product p53 has been described to interact with the EGFR/KRAS signaling pathway in PDA[34,40,41]. Additionally, p53 was recently also found to potentially serve as an independent predictive biomarker for treatment success with the monoclonal anti-EGFR antibody cetuximab in locally advanced rectal cancer[42]. Pre-liminary findings from 50 patients treated within AIO-PK0104 showed that overall survival was independent of p53 expression; however, progression free survival was significantly reduced in patients with p53 loss (1.8 mo) or overexpression of p53 usually resulting in dominant negative p53 (2.5 mo) compared to normal levels of p53 expression (6 mo)[38]. These pre-liminary findings may provide further evidence that not only a loss of p53 but also its overexpression is an important step in carcinogenesis and might be correlated to poor prognosis as also suggested by other studies[43]. Further research in PDA is necessary to confirm these findings and to clarify whether the observed correlation is of prognostic or predictive nature.

Predictive biomarkers of the VEGF pathway

Angiogenesis inhibitors like the VEGF inhibitor bevacizumab have proven to be beneficial as add-on treatment in multiple cancer entities like colorectal and non-small cell lung cancer[44]. In PDA *antiangiogenic* treatment has failed to show a significant effect in unselected patient populations so far[8]. Lambrechts *et al*[44] identified a possible predictive biomarker to select patients who might benefit from anti-VEGF treatment with bevacizumab: using blood samples collected within the AViTA trial, they genotyped a set of 157 single nucleotide polymorphisms (SNP) in patients who had received gemcitabine and erlotinib plus either bevacizumab ($n = 77$ patients) or placebo ($n = 77$ patients). They identified the rs9582036 SNP in the VEGF receptor 1 region, which significantly correlated with progression-free and overall survival in the bevacizumab-treated group but not in the placebo group. Bevacizumab-treated AA carriers of the rs9582036 SNP showed a median overall survival of 10.2 mo (95%CI: 7.8-14.9) while AC and CC carriers showed a median overall survival of 5.9 mo (95%CI: 4.0-11.5) and 4.7 mo (95%CI: 4.3-NA), respectively. Using a novel multiplex ELISA system Nixon *et al*[20] recently analyzed 31 different factors in plasma samples of 328 patients with metastasized or locally advanced PDA who had re-

ceived gemcitabine plus either bevacizumab or placebo within the CALGB 80303 study; after multivariate analysis three factors were identified as possible predictive biomarkers: while low levels of VEGF-D were found to be predictive for improved outcome in the bevacizumab group, below median levels of CXCL12 (SDF-1alpha) and angiopoetin 2 (Ang2) predicted a lack of benefit in the bevacizumab group. Further prospective biomarker studies are clearly necessary to confirm these pre-liminary findings and to assess the possible benefit of add-on treatment with bevacizumab in a pre-selected PDA population.

Biomarkers for the efficacy of gemcitabine
hENT1: Gemcitabine has been established as standard agent in the adjuvant and palliative chemotherapy setting of PDA more than a decade ago[45]. Gemcitabine uptake by PDA cancer cells is thought to be dependent on human equilibrative nucleoside transporter 1 (hENT1), suggesting hENT1 as a possible predictive biomarker for treatment response to gemcitabine[46,47]. In PDA patients receiving adjuvant treatment with gemcitabine or 5-FU within the RTOG 97-04 study, Farrell *et al*[48] indeed demonstrated that patients treated with gemcitabine showed significant better overall survival if hENT1 was expressed in cancer cells as determined by immunohistochemistry in resected tumors (hazard ratio for death for hENT1 expression *vs* no hENT1 expression: 0.40; 95%CI: 0.22-0.75; $P = 0.03$). No correlation between overall survival and hENT1 expression was found in the 5-FU treated group indicating that hENT1 is a predictive biomarker for treatment response to gemcitabine. In line with these recent findings, Morinaga *et al*[49] previously reported superior overall survival for patients with high levels of hENT1 (22.2 mo) *vs* patients with low hENT1 levels (11.8 mo) in a population that had received adjuvant gemcitabine chemotherapy. In the largest retrospective study to date, Maréchal *et al*[50] collected tumor samples from 434 surgically resected PDA patients among whom 243 had received gemcitabine-based regimens. They found that high hENT1 expression was a strong predictive factor for superior overall survival in the gemcitabine treated group (hazard ratio for death high *vs* low hENT1 expression = 0.43; 95%CI: 0.29-0.63; $P < 0.0001$). In a recent review on 18 (mainly retrospective) clinical studies Jordheim and co-workers concluded that "it has been clearly shown that hENT1 expression is a predictive marker for patient outcome after (adjuvant) gemcitabine therapy" in resectable PDA[51]. This conclusion is also supported by the recently reported translational hENT1 data from the ESPAC studies: a retrospective subgroup analysis on 352 patients with resected PDA treated with either adjuvant 5-FU or gemcitabine found that hENT1 serves as a predictive marker for the efficacy of gemcitabine but not for 5-FU[52]. To overcome the poor prognosis in low hENT1 expressing PDA, CO-101, a chemically modified gemcitabine molecule thought to be capable of entering the cell independent of hENT1, was developed[53]. In

the 'low hENT1 and adenocarcinoma of the pancreas (LEAP)' trial, the efficacy and safety of CO-101 was investigated in chemo-naive metastatic PDA patients. Enrolling 360 patients, this international randomized phase II trial was also the first to prospectively assess the value of hENT1 as a predictive biomarker for treatment response to gemcitabine. Astonishingly, the LEAP trial not only demonstrated that CO-101 had no additional value over standard gemcitabine treatment, it also indicated that hENT1 expression does not correlate with overall survival in gemcitabine-treated patients with metastatic PDA[53]. Further research will have to elucidate whether these contrasting findings are due to different research approaches (retrospective *vs* prospective studies), differences in methodology (*e.g.* use of different antibodies) or if hENT1 expression has differing functions as a biomarker in the adjuvant and metastatic PDA setting.

RRM1 and dCK: Several other molecules involved in the metabolism of gemcitabine are currently under investigation as potential biomarkers in PDA: retrospective evidence suggests that - besides hENT1 - also deoxycytidine kinase (dCK) may be able to predict benefit from adjuvant gemcitabine in resected PDA[54]. dCK is responsible for the intracellular phosphorylation of the prodrug gemcitabine to its mononucleotide in a rate-limiting manner. Thus high expression levels of dCK may enhance the efficacy of the drug. RRM1 (ribonucleotide reductase M1) is a cellular target for gemcitabine and may additionally also act as a tumor suppressor. Preliminary evidence in resectable PDA showed that RRM1 may potentially serve as both a prognostic (in non-gemcitabine treated patients) and a predictive (in gemcitabine treated patients) biomarker[54].

Biomarker for the efficacy of nab-paclitaxel
SPARC: Adjacent stromal tissue is a hallmark of PDA believed to be an important contributor to poor treatment outcome by reducing drug delivery to cancer cells[55]. New treatment strategies aim on facilitating drug delivery to cancer cells by reducing tumor stroma. A promising candidate is the recently FDA-approved albumin bound nab-paclitaxel, which was originally developed to avoid toxicities observed in treatment with solvent-based paclitaxel[9]. In addition to a favorable safety profile, nab-paclitaxel has been shown to deplete tumor stroma and increase intratumoral gemcitabine concentration by a factor of 2.8 in mice bearing xenograft PDA tumors[56]. Further, co-administration of gemcitabine and nab-paclitaxel reduced levels of the gemcitabine metabolizing enzyme cytidine deaminase, making PDA cells more sensitive to gemcitabine treatment[9]. Recent results from the phase III 'metastatic pancreatic adenocarcinoma clinical (MPACT) trial showed a statistically significant increase in overall survival from 6.7 mo in patients receiving single-agent gemcitabine to 8.5 mo in patients receiving the combined nab-paclitaxel/gemcitabine regimen[57]. Recent findings in humans confirmed that

stromal depletion by nab-paclitaxel might be responsible for the reported survival benefit[58]. For intracellular uptake of nab-paclitaxel into PDA stromal cells, specific albumin-binding proteins are necessary. Secreted protein acidic and rich in cysteine (SPARC) has been proposed to be one of them[9]. SPARC is a matricellular glycoprotein involved in different biological processes like wound repair or angiogenesis[59]. Its overexpression and correlation with poor prognosis independent of the therapeutic agent has been described in different human cancers like colon, esophageal, breast and lung cancer[60]. In PDA it was demonstrated that increased SPARC expression in adjacent fibroblasts but not in cancer cells conversely correlates to overall survival[60]. Results from a phase I / II nab-paclitaxel trial suggested that elevated SPARC levels in fibroblasts adjacent to PDA might be a predictive marker for treatment success with nab-paclitaxel[56,61]. Yet a very recent study using SPARC knockout mice reported drug delivery and antitumoral effects of murine nab-paclitaxel to be independent of SPARC expression[62]. Thus further research will be necessary to elucidate the potential use of SPARC as a predictive biomarker for nab-paclitaxel treatment in humans; specifically the translational results on SPARC from the large international MPACT study are urgently awaited in this context.

Future directions

Translational research studies conducted so far have failed to identify reliable prognostic or predictive biomarkers for PDA. Besides methodological limitations like retrospective design and heterogeneous study populations, most trials focused only on specific mutations in a small number of genes. Yet prognosis and treatment response might depend on the interaction of a large variety of genes and mutations as proposed in a work of Collisson *et al*[63]. In this study microdissected DNA of resected PDA was analyzed using gene expression microarray analysis. A 62-gene signature for PDA was defined by means of different statistic models. Subsequently tumor probes were divided into three different subgroups depending on their genetic signature. Subgroups were classical type for PDA expressing high levels of adhesion-associated and epithelial genes, quasi-mesenchymal type for PDA expressing high levels of mesenchyme-associated genes and exocrine-like type for PDA expressing high levels of tumor cell derived digestive enzyme genes. Prognosis between these three subtypes differed significantly with classical type having the best and quasi-mesenchymal type having the worst prognosis regarding overall survival. In further experiments Collisson *et al*[63] analyzed human and murine PDA cell lines using the 62-gene microarray technique. Dependence on KRAS was analyzed using RNAi. Proliferation of classical subtype PDA cell lines was more prone to inactivation of KRAS by RNAi (of note this approach did not distinguish between wildtype and mutant KRAS alleles). Additionally, classical PDA cell lines were more sensitive to treatment with erlotinib while quasi-mesenchymal cell lines were more sensitive

to gemcitabine[63]. Further clinical research is required to translate these findings into clinical practice. As cancer genome sequencing becomes more available and less expensive[64], analyzing large subsets of genes appears to be a promising future approach to predict treatment response and prognosis in PDA.

CONCLUSION

In this topic highlight several potential biomarkers for prognosis, tumor biology and treatment response of PDA were identified and discussed (as summarized within Table 1). Despite promising pre-liminary results, translational research has failed to establish reliable biomarkers for clinical practice so far. Main limitations for most trials on potential biomarkers conducted in PDA were: non-comparable patient cohorts, retrospective design and non-consistent treatment protocols and molecular methods used. Besides the general need for more accompanying translational studies in pancreatic cancer trials, future studies on potential biomarkers should be conducted prospectively in well-defined patient populations, using standardized molecular methods and profound biostatistical analysis. Furthermore, innovative technologies like cancer genome sequencing and multiplex ELISA platforms might help to identify new options in predicting prognosis and facilitating treatment allocation in PDA.

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2014 ADVANCES IN PANCREATIC CANCER

Genetic predisposition to pancreatic cancer

Paola Ghiorzo

Paola Ghiorzo, Department of Internal Medicine and Medical Specialties, University of Genoa and IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazioanle per la Ricerca sul Cancro, 16132 Genoa, Italy
Correspondence to: Paola Ghiorzo, PhD, Department of Internal Medicine and Medical Specialties, University of Genoa and IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazioanle per la Ricerca sul Cancro, V.le Benedetto XV 6, 16132 Genoa, Italy. paola.ghiorzo@unige.it
Telephone: +39-10-3538949   Fax: +39-10-3537543

Abstract

Pancreatic adenocarcinoma (PC) is the most deadly of the common cancers. Owing to its rapid progression and almost certain fatal outcome, identifying individuals at risk and detecting early lesions are crucial to improve outcome. Genetic risk factors are believed to play a major role. Approximately 10% of PC is estimated to have familial inheritance. Several germline mutations have been found to be involved in hereditary forms of PC, including both familial PC (FPC) and PC as one of the manifestations of a hereditary cancer syndrome or other hereditary conditions. Although most of the susceptibility genes for FPC have yet to be identified, next-generation sequencing studies are likely to provide important insights. The risk of PC in FPC is sufficiently high to recommend screening of high-risk individuals; thus, defining such individuals appropriately is the key. Candidate genes have been described and patients considered for screening programs under research protocols should first be tested for presence of germline mutations in the BRCA2, PALB2 and ATM genes. In specific PC populations, including in Italy, hereditary cancer predisposition genes such as CDKN2A also explain a considerable fraction of FPC.

Core tip: Pancreatic adenocarcinoma is the most deadly of the common cancers. Identifying families with hereditary pancreatic cancer can aid appropriate selection of individuals who are at high risk and are good candidates for prevention and screening programs. Although genetic predisposition to pancreatic cancer remains largely unexplained, next-generation sequencing is likely to provide important insights. Candidate genes have been described and patients considered for screening protocols should first be tested for germline mutations in these genes. In specific pancreatic cancer populations, including Italy, hereditary cancer predisposition genes such as CDKN2A also explain a considerable fraction of hereditary pancreatic cancers.

INTRODUCTION

Pancreatic adenocarcinoma (PC) is the deadliest among the common cancers. Its incidence is on the rise, especially in North America, Japan and Europe, where it represents the fourth to fifth most frequent cause of cancer mortality. Despite advances in therapy, diagnostic imaging and understanding of genetic factors, PC mortality rates have not declined appreciably in the past 20 years, and PC mortality still nearly equals its incidence (roughly

280000 new cases per year, 7000 of which are in Italy), leading to an estimated 227000 deaths per year worldwide[1-4]. The only potentially curative treatment for PC is surgical resection. Median survival following resection ranges from 13 to 21 mo, while without surgery, median survival is a mere 2.5-8 mo[5,6]. However, as most PCs are diagnosed late, and < 5% of tumors are resectable at the time of diagnosis, 5-year survival for PC remains low (< 5%). Early detection of stage 1 disease with curative resection has been shown to improve 5-year survival rates upwards to 60%[7].

The incidence of PC in the general population is not as high as that of other more common cancers (*e.g.*, colorectal cancer), therefore, nonselective screening is not recommended. However, targeted screening may hold promise for high-risk individuals (HRIs) identified by their family history or because of a known genetic predisposition. To date, no standard diagnostic approach or early detection method for PC has been developed, and screening remains challenging[8]. Accurate risk stratification and correct identification of HRIs with a genetic predisposition to the disease who may benefit from prevention and screening interventions in high-volume centers with ongoing research programs on PC[8,9] is thus crucial.

In recent years some excellent reviews have described susceptibility genes for PC, its biology and screening intervention protocols[8-12]. The aim of this review is to provide an update of recent findings on genetic susceptibility to PC, describing standard and novel approaches for the identification of susceptibility genes, as well as genetic data recently obtained for the first time in the Italian PC population.

RISK FACTORS FOR PANCREATIC ADENOCARCINOMA

Biological, lifestyle and environmental risk factors
PC incidence shows wide variations across countries, suggesting that biological, lifestyle and environmental factors are involved in determining increased PC risks, which range between two- and 13-fold[4]. PC is age dependent; in the United States, the median age at diagnosis is 72 years. Only 5%-10% of patients with PC develop the disease before the age of 50 years, and these are likely to include patients with an underlying genetic predisposition or who have previously undergone radiotherapy[4]. Sex and race also play a role, probably related to differences in smoking rates in men, as well as race-specific genetic differences in the ability to detoxify tobacco products, or vitamin D deficiency in blacks. Although these factors cannot be modified, lifestyle and environmental risk factors are controllable, cause 20%-25% of all PCs, and are thus of importance for HRIs. Heavy alcohol intake is associated with a modest increased risk of PC, while chronic pancreatitis, long-term diabetes, *Helicobacter pylori* infection, overweight, vitamin D deficiency and occupational exposures are associated with significantly increased risk. Conversely, atopic allergy and

use of metformin to treat diabetes have been associated with a reduced risk.

Susceptibility genes in HRIs
Although lifestyle modifications are possible and may help reduce PC risk, high-risk factors are not controllable and are the ones that typically characterize candidates for prevention and screening interventions.

PC has a familial basis in as many as 10% of patients. Some of the familial aggregation of PC is due to chance, and some to shared environmental exposure such as cigarette smoking[6]. An inherited predisposition to PC is seen in a range of clinical settings. Several hereditary cancer syndromes are known to be associated with an increased risk of PC, mainly Peutz-Jeghers syndrome (PJS), melanoma pancreatic-cancer syndrome (MPCS) or familial atypical multiple mole melanoma (FAMMM)-PC, hereditary breast-ovarian cancer (HBOC), and to a lesser extent Lynch syndrome (LS) and familial adenomatous polyposis (FAP). In addition, an increased risk of PC is present in patients with hereditary pancreatitis or cystic fibrosis.

Approximately 20% of hereditary cases of PC are currently attributed to a known genetic syndrome. The term familial pancreatic cancer (FPC) applies to the remaining 80% of patients with an inherited predisposition: families in which at least two first-degree relatives (FDRs) have been diagnosed with PC but that do not meet the diagnostic criteria for the previous settings[13-16] (Table 1).

Genes for known hereditary cancer syndromes
As mentioned earlier, PC is known to occur in a range of hereditary diseases and syndromes.

PJS is an autosomal dominant hereditary disease with characteristic hamartoma polyps of the gastrointestinal tract, and mucocutaneous melanin pigmentation. Almost half of all PJS patients harbor germline STK11/LKB1 gene mutations. Affected individuals have a 36% cumulative lifetime risk of developing PC[17].

FAMMM is an autosomal dominant disease that is characterized by the occurrence of > 50 atypical nevi and malignant melanoma in two or more first- or second-degree relatives. Malignant melanoma, however, may also be familial in the absence of the FAMMM phenotype. Approximately 10% of melanomas have a familial aggregation pattern and mutations in the CDKN2A tumor suppressor gene are identified in roughly 40% of these families[18]. PC has been observed in a considerable proportion of kindreds with CDKN2A mutations. This is considered to be a distinct hereditary cancer syndrome, is termed FAMMM-PC or MPCS, and has been found to confer a 17% cumulative lifetime risk of developing PC. CDKN2A germline mutations account for 30%-40% of patients affected by MPCS or the FAMMM-PC syndrome[18-29].

HBOC is another autosomal dominant hereditary cancer syndrome and is caused by germline mutations in

Table 1 Syndromes and genes associated with hereditary predisposition to pancreatic adenocarcinoma, relative and lifetime risk

Settings of hereditary PC	RR of PC (-fold)	Cumulative lifetime risk by age 70 (%)	Genes identified
FPC syndrome			PALLD, CDKN2A, BRCA2, PALB2, ATM,....?
FDR with PC	2-3	2	
FDRs with PC	6	8-12	
or more FDRs with PC	14-32	40	
Hereditary cancer syndromes			
PJS	132	36	STK11/LKB1
MPCS/FAMMM	13-47	17	CDKN2A
HBOC	3.5-10	3-8	BRCA1, BRCA2
LS	8.6	< 5	MLH1, MSH2, MSH6
FAP	2-3	< 5	APC
Syndromes of chronic inflammation			
HP	50-80	40	PRSS1, SPINK1
CF	5	< 5	CFTR

HP: Hereditary pancreatitis; FAP: Familial adenomatous polyposis; PC: Pancreatic adenocarcinoma; FDR: First-degree relative; PJS: Peutz-Jeghers syndrome; MPCS: Melanoma pancreatic-cancer syndrome; FAMMM: Familial atypical multiple mole melanoma; HBOC: Hereditary breast-ovarian cancer; LS: Lynch syndrome; FPC: Familial PC; CF: Cystic fibrosis; HP: Hereditary pancreatitis.

the BRCA1 and BRCA2 genes. BRCA2 mutation carriers have an increased risk of breast, ovarian, and prostate cancer, as well as a 3.5-10-fold increased risk of PC[30,31], while the reported risk of PC for BRCA1 mutation carriers is about 2.5 times that of the normal population[32].

PC is also typical of LS, alternatively termed hereditary non-polyposis colorectal carcinoma syndrome. This syndrome is caused by mutations in the mismatch repair (MMR) genes MSH2, MLH1, MSH6 and PMS2. Individuals with mutations in the MMR genes have a risk of developing PC that ranges between 5% and 10%[33]. According to a recent study[34], PC risk is increased sevenfold in both MLH1 and MSH2 carriers belonging to LS families, especially at young ages, as noted by Lynch et al[35] as early as 1991.

Patients with FAP also have an increased risk of developing PC, with a relative risk of 4.6 (95%CI: 1.2-11.4)[36,37]. Finally, PC also occurs, if less frequently, in patients affected by Li-Fraumeni syndrome and ataxia telangiectasia.

Hereditary cancer syndromes in Italian PC patients

One of the difficulties in confirming that PC is a component of an inherited syndrome caused by germline mutations in a susceptibility gene is the lack of DNA from PC patients in families, which makes it impossible to conduct co-segregation analysis.

We recently investigated the contribution of hereditary cancer syndromes to PC in a hospital-based series of 225 Italian PC patients who were consecutively recruited at our center. Among these patients, 24% of those who presented with features suggestive of HBOC were BRCA1 or BRCA2 positive, and 10% of those who were suspected to be affected by LS carried mutations in the MMR genes[38,39]. Interestingly, 45% of the cases suspected for MPCS were found to harbor mutations in CDKN2A[40-42]. This result corroborates previous findings on the high occurrence of PC in Italian melanoma families with CDKN2A mutations[27,43-46]. The presence of CDKN2A mutations in PC patients selected from a case-control series

shows that an unbiased association exists between PC and CDKN2A germline mutations. No other hereditary syndromes were observed in this series that could drive selective screening of other genes.

Genes for hereditary conditions associated with PC

Hereditary pancreatitis: Hereditary pancreatitis (HP) is currently considered to be an independent nosological unit. It is an autosomally dominant disease with 80% penetrance. In patients with HP, trypsin becomes activated while still in the pancreas. This leads to partial digestion of the pancreatic tissue, causing inflammation.

A strong genetic association exists between HP and germline mutations in the PRSS1, SPINK1 and CFTR genes[47]. Patients with HP have an about 80% relative risk and a 40% lifetime risk of developing PC. If these individuals are smokers, then PC develops, or rather is diagnosed, up to two decades earlier than in non-smokers. Similarly, alcohol consumption also leads to a 20-year earlier diagnosis of PC[48,49].

Cystic fibrosis: Cystic fibrosis (CF) is an autosomal recessive disease that is caused by mutations in the *CFTR* gene. CF is characterized by the production of viscous mucus, which blocks the airways and leads to obstruction of the pancreatic duct, thus increasing the risk of inflammation. Patients with CF are at increased risk of chronic pancreatitis and of PC[50].

FPC genes

FPC is mostly inherited in an autosomal dominant fashion, and presents with a heterogeneous phenotype. Prospective studies have reported an increased risk of developing PC in unaffected FDRs of PC patients, which depends on the number of relatives with PC in the family[51]. This risk has been estimated to be 6.4-fold greater in individuals with two FDRs with PC (lifetime risk 8%-12%) and 32-fold greater in individuals with three or more FDRs with PC (lifetime risk 40%) (Table 1). Among kindreds with FPC,

the risk is higher in kindreds with young-onset PC (age < 50 years, relative risk = 9.3) compared with kindreds without young-onset PC[15,52,53]. Furthermore, evidence indicates that the risk of PC is modestly increased in FDRs of patients with sporadic PC compared to the general population[53], in which the lifetime risk of developing PC is slightly less than 1% (0.5% at age 70 years). Anticipation has been described in 59%-85% of FPC families; indeed, patients in younger generations are affected by the disease about 10 years earlier than their affected relatives[54,55].

Studies focusing specifically on FPC genes have not been successful so far in clarifying the genetic basis of the disease[8,15,52]. Several genes underlying susceptibility to the cancer syndromes associated with PC have been investigated for their involvement in FPC predisposition. Although the genes responsible for PJS[56] and LS[57,58] do not seem to play a major role, BRCA2 and BRCA1 are interesting candidates.

BRCA2 has been considered an important PC predisposition gene since its discovery[59], and recent reports have estimated that it accounts for 6%-12% of FPC families[8,60,61]. BRCA1 gene mutations have been reported in a small number of patients with FPC[62,63]. Increasing evidence is emerging that points to CDKN2A as an FPC susceptibility gene[42,64] and other, novel candidate genes (and loci) are being discovered. Indeed, over the past decade, FPC families have been found to harbor mutations in several different genes.

PALLD: In 2002, linkage analysis of a large FPC pedigree from the United States showed significant linkage to chromosome 4q32-34[65]. Four years later, an oncogenic germline mutation at this locus, in the Palladin (*PALLD*) gene, which encodes a cytoskeletal protein, was identified in affected members of that family. It was therefore suggested that PALLD may be a major PC susceptibility gene[66]. But this hypothesis was not supported by later studies on Italian FPC families and on families from other European countries[42,67,68].

BRCA1 and BRCA2: Although germline mutations in the *BRCA1* gene have been reported in a small number of patients with FPC[62,63], mutations in BRCA2 have long been reported to be the most frequently identified genetic alterations in FPC. Early studies with small sample sizes found BRCA2 mutations in 15% of FPC families from Germany and the United Kingdom and in 17% of families from North America[59,60]. These results however could not be confirmed in larger cohorts, in which deleterious BRCA2 mutations were detected in 6% of moderate- and high-risk FPC families[58,61]. BRCA2 deficiency in PC seems to be of clinical importance, because PCs in BRCA2-positive patients are characterized by marked sensitivity to poly (ADP-ribose) polymerase inhibitors and mitomycin[69-71].

We recently assessed the role of BRCA1 and BRCA2 as FPC susceptibility genes in the Italian population[42]

and found no germline mutations.

PALB2: PALB2, which binds to the BRCA2 protein, was reported to be a new PC susceptibility gene after whole genome sequencing identified truncating PALB2 mutations in 3.1% of a series of North American FPC patients[72]. PALB2 mutations were later detected in 3.7% of German and British FPC families[73]. Conversely, a Dutch study on 28 FPC families identified no mutations in PALB2[74]. These findings suggest that PALB2 mutations may explain FPC in a small subset of European families, especially in those with an additional occurrence of breast cancer. Indeed, PALB2 is increasingly considered a good candidate for clinical testing in BRCA1- and BRCA2-negative HBOC families[75]. PALB2 testing in a series of Italian PC patients suspected for HBOC described above, and in FPC patients, yielded no mutations, despite the fact that we screened the gene both by Sanger sequencing and by multiplex ligation-dependent probe amplification assay, in order to rule out large genomic rearrangements[38,42].

Germline mutations in other genes in the BRCA2 pathway, namely FANC-C and FANC-G, have been linked to early-onset PC, but segregating germline mutations in these genes have yet to be identified in FPC families[76].

ATM: Recently, heterozygous germline mutations in the ataxia telangiectasia mutated (ATM) gene have been identified in two kindreds with FPC[77]. Subsequent analysis of 166 additional FPC patients identified another four deleterious ATM germline mutations, while none were detected in 190 spouse controls. The prevalence of ATM mutations in the whole FPC cohort was 2.4% (4/166), and 4.6% (4/87) in families with three or more affected members[77]. These findings suggest that ATM mutations in these families may underlie PC, driven by the classic two-hit model for tumor suppressor genes.

CDKN2A: CDKN2A germline mutations account for 30%-40% of patients with MPCS or FAMMM-PC[18-29] and have generally been considered to play a minor role in FPC[61,78-80]. However, there is increasing evidence that CDKN2A (p16INK4a) mutations occur in FPC without metachronous or synchronous occurrence of melanoma in the family.

Two recent papers describing our Italian and the Dutch PC population suggest that CDKN2A may be an FPC susceptibility gene and that CDKN2A testing may be appropriate in FPC even when melanoma does not occur in the family[42,64]. Previously, a large North American study of 1537 unselected patients with PC found that 0.6% carried CDKN2A mutations. Among the 120 FPC cases in that study, four (3.3%) were CDKN2A positive. The authors concluded that screening of patients with PC for CDKN2A mutations should not be performed, but also that these mutations are especially penetrant among smokers[80].

Most CDKN2A mutations are missense mutations lo-

Table 2 Role of CDKN2A mutations in familial pancreatic adenocarcinoma and melanoma pancreatic-cancer syndrome

Study[1]	N° of FPC families	CDKN2A mutation found	Type of CDKN2A mutations	% of CDKN2A positive	N° of MPCS	CDKN2A mutation found	% of CDKN2A positive	Type of CDKN2A mutations
Slater et al[61] 2010; Bartsch et al[79] 2002	56	0	-	-	5	2	40	p.Q50X, p.E119X
McWilliams et al[80] 2010	119	3	c.-34G>T,p.V95fs, p.D153spl,	2.5	39	2	5.3	p.D153spl, pL16R
Ghiorzo et al[42] 2012	16	5	p.E27X,p.G67R, p.G101W, c. 201ACTC>CTTT	31	5	2	40	p.L65P, p.G101W
[2]Harinck et al[64] 2012	24	3	p.Ser8fs, p.Ala76fs	12	4	3	75	p.Ala76fs

[1]This table only includes studies that analyze CDKN2A mutations in pancreatic adenocarcinoma (PC) probands from familial PC (FPC) families comparing them with PC probands from Melanoma pancreatic-cancer syndrome (MPCS) families belonging to the same population. The prevalence of CDKN2A mutations in MPCS/FAMMM-PC e families, as analyzed in melanoma probands, is described in the text. [2]A melanoma was diagnosed in one FPC family after the proband was found to carry a mutation in CDKN2A.

cated in the coding sequences of exons 1 and 2, common to both the tumor suppressors encoded by this locus (p16INK4a and p14ARF). A number of these mutations seem to derive from ancestral founders[81]. We previously performed germline testing of CDKN2A in a series of unselected PC patients and found that 4% of these patients were CDKN2A positive[40,41]. In a subsequent study we extended the analysis to 225 PC patients and controls. The CDKN2A mutation rate in the 225 PC cases was 5.7%, ranging from 2.6% in patients without a family history of PC or melanoma, to 17% when two cancers occurred in the index patient or FDRs, and to 45% when three or more cancers occurred. Interestingly, 25% of the cases with one FDR with melanoma were mutation positive. Sixteen probands of FPC families were identified, defined for having at least two FDRs affected by PC, and no other manifestation of a hereditary cancer syndrome, or melanoma. Deleterious or potentially deleterious CDKN2A mutations were found in five of the probands (31%)[42]. The mutation frequency ranged from 20% in FPC families with two affected members to 50% in families with three, and was comparable to the mutation rate in melanoma families[46] (Table 2).

Within the PC families with no CDKN2A mutations, anticipation was observed, which is consistent with previous studies that reported anticipation for BRCA2 carriers in FPC families without CDKN2A mutations[55].

The CDKN2A mutation rate in our FPC cases was nearly 10 times that observed in the North American study by McWilliams and colleagues[80]. This result indicates that a sizeable subset of Italian FPC families may carry CDKN2A mutations, and likely reflects the prevalence of founder mutations in CDKN2A in our population[43-46,82,83].

Approaches to CDKN2A genetic testing

It has been proposed that individuals should be referred for CDKN2A testing when at least one of the following conditions is met: (1) a personal history of melanoma and an FDR with melanoma; (2) more than two confirmed primary melanomas; (3) more than three (first-degree or second-degree) relatives with melanoma; (4) a personal or a family history of PC and melanoma; (5) a personal history of melanoma; and (6) a personal and/or a family history of atypical moles[13]. Other recommendations have included patients with more than three melanomas, or families with at least one melanoma and two other instances of melanoma or PC in the family, with mutation yields ranging between 20% and 40%[84].

Had we followed these criteria we would have identified two out of five (40%) of our mutation-positive families with both melanoma and PC. However, as none of the criteria include FPC families, we would not have identified the CDKN2A-positive FPC kindreds. The North-American study mentioned earlier came to the same conclusion, because the majority of their mutations were identified in FPC families, despite their low overall mutation frequency[80]. Their finding is probably more generalizable than ours, both because of their sample size and because it was not influenced by the presence of founder mutations.

Taken together, our results confirm that the occurrence of at least three cancer events (including PC and melanoma) in the family is a good predictor of CDKN2A mutations (45%). Importantly, however, the likelihood of identifying a CDKN2A mutation may also be high in families with two or more instances of PC or with one instance of PC and one of melanoma among FDRs, because we found that 17% of such kindreds were positive for CDKN2A mutations[42].

Harinck and colleagues also performed CDKN2A mutation analysis in 28 FPC families. Unlike ours, their selection criteria included presence of melanoma, and indeed melanoma also occurred in four of their families (14%), Interestingly, CDKN2A mutations were identified in three of these melanoma-positive families, confirming that CDKN2A mutations are frequently found in families affected by both PC and melanoma. The prevalence of CDKN2A mutations in their FPC families with no occurrences of melanoma was 12% (n = 3). These CDKN2A-positive families would not have been identified had the recommendations mentioned above

been followed, which are based on studies that found no CDKN2A mutations in FPC families without melanoma[13,26,61,79]. The prevalence of CDKN2A mutations in the FPC families studied by Harinck *et al*[64] may have actually been underestimated. Indeed, affected relatives in some of the families in that study were unavailable for DNA testing, so unaffected FDRs were tested instead. In such cases a negative test does not rule out the presence of a pathogenic mutation unless a specific mutation has been found in another relative.

Harinck and colleagues concluded that CDKN2A mutations are found in a considerable proportion of families with FPC (Table 2), and therefore CDKN2A mutation analysis should be performed in FPC families even in the absence of reported melanomas. According to the authors this strategy will enhance the recognition of individuals at risk for PC and facilitate the early detection of melanomas.

A number of reports have suggested that BRCA2 mutation analysis should be performed in FPC families that do not meet the criteria for HBOC[59,61]. Similarly, our findings and those reported by Harinck and colleagues emphasize the need to include CDKN2A mutation analysis in genetic testing for FPC families, even in the absence of reported melanomas[42,64].

Novel predisposition genes: evidence from NGS and genome-wide association studie

The discovery of additional FPC genes is one of the most exciting opportunities in PC research. As the speed and ease of testing increase and costs fall as a result of NGS, we expect that a number of new FPC genes will be discovered in the coming years. Exome sequencing has already led to the identification of PALB2 and ATM mutations in FPC, and much hope is being placed in postgenomic studies[72,77]. Indeed, recent genome-wide association studie (GWAS) and post- GWAS analyses have identified chromosome regions containing novel susceptibility loci for PC.

One such study, the PanScan Project, has identified several common polymorphisms affecting PC susceptibility. In that study, single nucleotide polymorphisms (SNPs) in ABO, sonic hedgehog (SHH), telomerase reverse transcriptase, nuclear receptor subfamily 5, group A, member 2 were found to be associated with PC risk. The scan also identified loci on chromosomes 13q22.1 and 15q14, to which no known genes or other functional elements are mapped[85,86].

Another GWAS on PC risk has been performed in the Japanese population[87], and yielded three new loci on chromosomes 6p25.3 (SNP rs9502893, 25 kb upstream of FOXQ1), 12p11.21 (SNP rs708224, in the second intron of BICD1) and 7q36.2 (SNP rs6464375, in the first intron of DPP6). Another still has been conducted in the Chinese population[88] and identified five novel PC susceptibility loci at chromosomes 21q21.3 (SNPrs372883, in the 3' UTR of gene BACH1), 5p13.1 (SNP rs2255280, in intron 1 of gene DAB2), 21q22.3 (SNP rs1547374,

upstream of gene TFF1), 22q13.32 (SNP rs5768709) and 10q26.11 (SNPrs12413624). The latter two SNPs are not located in the immediate vicinity of any gene.

Several recent reports have also shown associations between other genetic variants and PC risk and progression, and their impact on survival is currently being investigated[89-91].

The ABO gene in particular has been further investigated, and a link between ABO blood type and PC has been established. Non-O blood types have been found to account for 17% of all new PC cases, showing a protective effect of the O blood group. However, the exact mechanism that links PC and blood group remains unclear[92]. Whether genetic variability at the ABO locus may be involved in PC survival is currently under investigation[93].

Recent analysis of GWAS data has revealed that two pathways, the neuroactive ligand receptor interaction and olfactory transduction, are significantly associated with PC risk, and has shown that four genes are significantly associated with PC risk, adding OR13C4 to the previously identified ABO, HNF1A and SHH[2-4] genes. These findings provide new insights into the polygenic basis of PC susceptibility and etiology[94].

Gene-environment interaction

Among PC families, the risk of developing PC is higher in younger subjects and is likely modified by nongenetic risk factors such as exposure to tobacco smoke. Not only do smokers have a 2-3-fold greater risk of developing the disease compared to non-smokers, but they generally develop the disease at an earlier age[95,96].

An interesting example of gene-environment interaction for PC was shown for germline CDKN2A mutations in the large North American hospital-based study mentioned earlier, which investigated both the prevalence of germline mutations in PC patients, and their penetrance[80]. The authors found that penetrance for PC and melanoma was increased among mutation carriers, with PC risk estimates of 58% (95%CI: 8%-86%) by age 80 years and melanoma risk estimates of 39% (95%CI: 0%-80%) by age 80 years. Among ever-smokers, the risk of PC was higher for CDKN2A mutation carriers compared to non-carriers (HR = 25.8, $P = 2.1 \times 10^{-3}$), but among non-smokers the comparison did not reach statistical significance. The authors concluded that CDKN2A mutations in PC patients are rare but notably penetrant, and that CDKN2A mutation carriers, as well as being candidates for prevention and screening studies, should be counseled to avoid tobacco use.

Identification of HRIs: in silico analyses, genetic testing, role of registries

Genetic testing can identify a family's underlying genetic susceptibility to PC, but has limited scope because the genetic basis of much of the inherited susceptibility to this disease remains unexplained. Additional PC susceptibility genes may be discovered in the near future that should improve our ability to identify individuals who

Table 3 Proposed inclusion criteria for pancreatic adenocarcinoma screening programs in high-risk individuals, identified based on family history and possibly on genetic background

Current (based on family history alone or on genetic background):
Family history:
  Three or more relatives in the same lineage affected by PC
  Two relatives affected by PC, at least one of which is a FDR of the individual
  Hereditary pancreatitis
  > 10-fold increased risk as established by PancPRO
Genetic background:
  Germline carrier of a mutation in a candidate gene with at least one FDR or SDR affected by PC
  Mutation-positive individual in a PJS kindred
Proposed (based on family history and genetic background):
Family history: Identification of a hereditary syndrome or a 10-fold increased risk established by PancPRO
Genetic background: According to testing in candidate genes (CDKN2A, BRCA1-2, ATM, PALB2, STK11, PRSS1, SPINK1…)
  Mutation identified: Propose screening to carriers of germline mutation
  No mutation identified: Propose screening to all HRIs
In populations with a high prevalence of germline mutations in candidate genes (e.g., CDKN2A founder mutations in Italy or the Netherlands)
The same as above + test candidate genes according to specific genetic background, even in the absence of all criteria for hereditary syndromes or of a PancPRO score > 10

PC: Pancreatic adenocarcinoma; FDR: First-degree relative; PJS: Peutz-Jeghers syndrome; HRIs: High-risk individuals.

would benefit most from pancreatic screening in the context of research protocols[11].

Family history remains the main tool to quantify PC risk. Risk stratification is determined by the number of affected individuals in the family and the degree of relatedness between those individuals and other family members. The phenotypic variance seen in FPC families and the heterogeneity of the hereditary cancer syndromes potentially involved require careful study of the family tree over at least three generations, and histopathological confirmation of all diagnoses.

A computer-based risk assessment tool, PancPRO (http://astor.som.jhmi.edu/BayesMendel/pancpro.html), which uses this type of information has been shown to provide an approximate risk assessment for FPC families[97,98]. Families with high PancPRO scores would generally be identified by standard criteria, but PancPRO has the advantage that it can assign a quantitative risk score to any family member, which also depends on the age at diagnosis (or death) of the affected relatives. PancPRO provides useful information about an individual's PC risk before he or she decides to undergo invasive screening. That information can also help identify appropriate candidates for research on screening protocols or genetic susceptibility. Indeed, according to a recent position paper by the Italian PC Registry, having a PancPRO risk score > 10 is one of the criteria for enrollment in screening programs for PC[99].

It is in the framework of these programs that our findings will be of value to establish the most appropriate criteria to select families for CDKN2A testing in Italy. We found that about 30% of our FPC patients with no occurrence of melanoma among relatives carried mutations in CDKN2A, and similar results have been reported in the Netherlands, therefore, we suggest that individuals considered at high risk because of their family history should undergo genetic testing for CDKN2A before they are enrolled in research surveillance programs,

especially in populations such as these, in which founder effect CDKN2A mutations are predominant (Table 3).

Genetic testing for hereditary PC mandates full informed consent as recommended by national guidelines for genetic testing for cancer susceptibility[100] and should be initially performed in affected individuals[11,13]. Germline genetic testing of patients with PC is currently underused, not least because clinicians often fail to take a detailed history of cancer occurrence in the family. The possibility that a hereditary cancer syndrome may be present in the family is therefore frequently overlooked. However, our data show that a considerable proportion of FPC families carry CDKN2A mutations, even in the absence of melanoma in the family.

A combination of risk prediction tool analysis and genetic testing is likely to be the most successful approach to identify HRIs (Table 3). Based on the results reviewed here, genetic testing should be performed after PancPRO analysis to stratify better risk and identify the HRIs who may benefit from PC surveillance programs performed in the context of research protocols.

More generally, affected members of FPC families should be analyzed for BRCA2, PALB2, ATM and CDKN2A mutations. Genetic analyses of other genes (e.g., LKB1 and BRCA1) should only be recommended if the family history is suggestive of the associated hereditary cancer syndromes[11].

CONCLUSION

PC remains one of the most challenging of all cancers. Numerous studies are currently under way to identify novel early detection tools for PC, and evidence is beginning to show that screening FDRs of individuals with several family members affected by PC can identify precursors of this malignant disease[8-13].

Prospective PC screening with endoscopic ultrasound, magnetic resonance imaging and magnetic resonance

cholangiopancreatography has been shown to detect precancerous lesions with a diagnostic yield ranging from 13%[101] to 76%[102], depending on study population (high or moderate risk, carriers or non-carriers of germline mutations), age at baseline screening, screening modalities, and definition of the diagnostic yield, with the highest yield obtained in confirmed carriers of CDKN2A germline mutations[103].

Appropriate inclusion of families at high risk of PC in registries provides an excellent tool to improve our clinical and genetic understanding of FPC[104]. Indeed, focused research projects can be conducted most efficiently when data from different FPC registries are combined.

Although much work is currently focused on clarifying the impact of common genetic variability on individual PC risk, much less is known about heritable susceptibility to PC compared to what is known about other heritable cancers. One viable option to expand our understanding of the genetic determinants of PC risk is to collect large sets of patients across different populations[91]. In this review we have described some important results on new susceptibility genes and loci that have been recently obtained by PC consortia[10].

Genetic risk factors are believed to play a major role, and several germline mutations have been identified that underlie hereditary susceptibility to PC in different settings, such as FPC and other hereditary cancer or chronic inflammation syndromes. The risk of PC in FPC is sufficiently high to recommend screening HRIs; therefore defining those HRIs appropriately is crucial.

In the general population, the lifetime risk of developing PC is 1%. Although they have a twofold increased risk of PC, the vast majority of individuals with a family history of PC will not develop the disease themselves. It is therefore important to explain the concepts of both relative and absolute risk to patients and their families. However, when an FDR of a PC patient is tested and found to carry a germline mutation in a high-risk gene, the risk is not negligible. Once penetrance and factors that modify penetrance have been taken into account, these individuals may be appropriate candidates for prevention or screening protocols, which should at all events only be directed at HRIs, defined to the best of our ability and possibly with genotypic data.

Utility analyses suggest that PC screening is most cost-effective in individuals whose lifetime risk of the disease is 16% or greater[9]. It can detect intraductal papillary mucinous neoplasm and pancreatic intraepithelial neoplasia, which are precursor lesions for FPC; importantly, the former are higher grade, more common, and multifocal in individuals with FPC compared with patients with sporadic PC[9].

In this review we emphasize the importance of testing CDKN2A in Italian patients with hereditary PC, even when there is no occurrence of melanoma in the family, in order to improve the accuracy of risk stratification and ensure appropriate selection of patients, which we think may be especially of value in populations with a high CD-

KN2A mutation rate (Table 3). Identifying high-risk family members is important to understand the biology of PC, to recommend risk reduction strategies and, in some cases, enrollment in cancer surveillance programs. Because the best methods for surveillance have yet to be established and given the overall complexities involved, HRIs and FPCs should be referred to, screened and managed by multidisciplinary teams with specific experience, in the context of research protocols at high-volume centers.

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Cancer stem cells: Involvement in pancreatic cancer pathogenesis and perspectives on cancer therapeutics

Cristiana Pistol Tanase, Ana Iulia Neagu, Laura Georgiana Necula, Cristina Mambet, Ana-Maria Enciu, Bogdan Calenic, Maria Linda Cruceru, Radu Albulescu

Cristiana Pistol Tanase, Ana Iulia Neagu, Laura Georgiana Necula, Cristina Mambet, Ana-Maria Enciu, Bogdan Calenic, Radu Albulescu, Department of Biochemistry-Proteomics, Victor Babes National Institute of Pathology, 050096 sect 5 Bucharest, Romania

Ana Iulia Neagu, Laura Georgiana Necula, Stefan S Nicolau Institute of Virology, 050096 sect 5 Bucharest, Romania

Ana-Maria Enciu, Bogdan Calenic, Maria Linda Cruceru, Carol Davila University of Medicine and Pharmacy, 050474 Sect 5 Bucharest, Romania

Radu Albulescu, National Institute for Chemical Pharmaceutical Research and Development, 050096 sect 5 Bucharest, Romania

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Correspondence to: Cristiana Pistol Tanase, MD, PhD, Department of Biochemistry-Proteomics, Victor Babes National Institute of Pathology, no 99-101 Splaiul Independentei, 050096 sect 5 Bucharest, Romania. bioch@vbabes.ro

Telephone: +40-21-31945 28   Fax: +40-21-31945 28

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Abstract

Pancreatic cancer is one of the most aggressive and lethal malignancies. Despite remarkable progress in understanding pancreatic carcinogenesis at the molecular level, as well as progress in new therapeutic approaches, pancreatic cancer remains a disease with a dismal prognosis. Among the mechanisms responsible for drug resistance, the most relevant are changes in individual genes or signaling pathways and the presence of highly resistant cancer stem cells (CSCs). In pancreatic cancer, CSCs represent 0.2%-0.8% of pancreatic cancer cells and are considered to be responsible for tumor growth, invasion, metastasis and recurrence. CSCs have been extensively studied as of late to identify specific surface markers to ensure reliable sorting and for signaling pathways identified to play a pivotal role in CSC self-renewal. Involvement of CSCs in pancreatic cancer pathogenesis has also highlighted these cells as the preferential targets for therapy. The present review is an update of the results in two main fields of research in pancreatic cancer, pathogenesis and therapy, focused on the narrow perspective of CSCs.

Key words: Cancer stem cells; Pancreatic cancer; Cancer stem cells signaling pathways; Targeted therapy; miRNA

Core tip: Pancreatic cancer is one of the most aggressive and lethal malignancies, despite remarkable progress in understanding pancreatic carcinogenesis and new therapeutic approaches. The presence of highly resistant cancer stem cells (CSCs) and the changes in their signaling pathways lead to drug resistance in pancreatic cancer. CSCs are considered responsible for tumor growth, invasion, metastasis and recurrence. CSC involvement in pancreatic cancer pathogenesis has also highlighted them as preferential targets for therapy. This review is an update of the results in two main fields of research in pancreatic cancer, pathogenesis and therapy, focused on the narrow perspective of CSCs.

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INTRODUCTION

Pancreatic cancer is one of the most aggressive and lethal malignancies. Despite remarkable progress in understanding pancreatic carcinogenesis at the molecular level as the identification of new therapeutic approaches, pancreatic cancer remains a disease with a dismal prognosis; the five-year survival rate is approximately 5%[1]. Although several histological subtypes of pancreatic cancer have been described, the most common form is pancreatic ductal adenocarcinoma. According to data published by the International Agency for Research on Cancer, pancreatic cancer death is the eighth or ninth most frequent cause of cancer death worldwide and is the fourth or fifth most common cause of cancer death in developed countries[2,3].

The main risk factors for pancreatic cancer include increasing age, smoking[4], chronic pancreatitis, diabetes mellitus, metabolic syndrome, low levels of serum vitamin D, family history of pancreatic cancer and rare inherited genetic conditions such as Peutz-Jeghers syndrome, familial melanoma and hereditary pancreatitis[5]. Age is a significant risk factor; the median age at diagnosis is 72 years. Pancreatic tumors are rarely diagnosed before the age of 50, and such cases are very likely to be associated with underlying predisposing genetic disorders. Approximately 5%-10% of pancreatic cancer patients report a family history of pancreatic cancer. The genes responsible for a minority of the familial clustering of pancreatic cancer have been identified, including STK11, CDKN2A, PRSS1, BRCA2 and PALB2[1,6].

The high mortality rate of pancreatic cancer is due to difficulty in early diagnosis[7,8] and its notorious resistance to chemotherapy and radiation[9]. Lack of clinical symptoms in early stages leads to delay in tumor detection; thus, approximately 80% of patients with pancreatic cancer have metastatic disease at the moment of diagnosis[10]. Existing systemic therapies for advanced disease are far from effective, and the median survival for patients with metastatic disease remains 6 mo. Surgery offers a better prognosis of a cure, but even those patients who undergo resection and receive adjuvant therapy have a median survival of 12-22 mo and a 5-year survival of 20%-25%[2,11].

Chemoresistance is a critical issue in pancreatic cancer. Among mechanisms responsible for drug resistance, the most relevant are changes in individual genes or signaling pathways, the influence exerted by tumor microenvironment (desmoplastic reaction) and the presence of highly resistant cancer stem cells (CSCs)[9]. The notion of CSCs has gained prominence, and several identified molecules and signaling pathways are relevant for the diagnosis and therapy of cancer. The paradigm of cancer-initiating stem cells has initially been developed with respect to blood cancers, where chronic conditions such as myeloproliferative neoplasms are due to mutations acquired in hematopoietic stem cells[12].

CANCER STEM CELLS IN PANCREATIC CANCER PATHOGENESIS

Cancer stem cells involvement in tumorigenesis

Pancreatic cancer (especially pancreatic ductal adenocarcinoma) is an aggressive malignancy, with one of the worst prognoses among solid tumors. Pancreatic cancer is typically diagnosed in late stages, when most patients are inoperable and when curable treatment is not available. Current therapies (radio- and chemotherapy) may improve prognosis and reduce tumor size but cannot target all pancreatic cancer cells[13,14].

Cancer stem cells, identified in a large number of human malignancies, represent 0.2%-0.8% of pancreatic cancer cells and are considered responsible for tumor growth, invasion, metastasis and recurrence[15]. Currently, there are two models that explain tumor development[16-18]. The stochastic model states that every cancer cell has the ability to initiate and promote tumoral growth. The other model, the "cancer stem cell hypothesis", proposes that tumor evolution is based on stem cells with a 'deregulated' self-renewal pathway. A recent and rapidly growing body of research shows solid evidence in support of the cancer stem cell model against the stochastic model[19,20]. The American Association for Cancer Research defines CSCs as cells within a tumor that have the capacity to generate the heterogeneous cancer cell lineages found in the tumor and that possesses the capacity to self-renew. CSCs also share other several important attributes: active telomerase expression, drug resistance to harming agents, the activation of antiapoptotic pathways, the ability to migrate and to metastasize and increased membrane transporter activity. To date, CSCs have been isolated and characterized only from a relatively small number of tumor types: breast, brain, pancreas, colon, blood and head and neck[21,22]. Several studies argue that cancer stem cells cannot be eradicated by current therapy and thus are responsible for tumor relapse and metastasis[23]. Many studies have demonstrated that multiple critical genes, including K-ras, p53 and p16, and key signaling kinases, such as PI-3K, mTOR, NF-κB, epidermal growth factor receptor (EGFR) and SHH, play important roles in pancreatic tumorigenesis[24].

Pancreatic cancer stem cells markers

Several pancreatic cancer stem cell (PCSC) subpopulations have been isolated using flow cytometry and combinations of positive and/or negative membrane surface markers[25-29]. Historically, research on stem cells and cancer stem cells from the hematopoietic system began long before studies in other tissues. As a result, several

markers identified in hematopoietic malignancies, such as cluster of differentiation (CDs), were also proposed as potential PCSCs markers. Li *et al*[30] were the first to identify a population of PCSCs using CD44, CD24 and ESA as separation markers. The cell fraction with the CD44+/CD24+/ESA+ phenotype exhibits several important cancer stem cell characteristics, including a minor population of cells (between 0.2% and 0.8%) that has the potential to form tumors in half of the mice used for transplantation. *In vitro* studies lend further support to arguments for the use of CD44 and CD24 as cancer stem cell markers. CD44+/CD24- cells isolated from PANC-1, a pancreatic adenocarcinoma cell line, exhibit a much higher tumorigenic potential than cellular subpopulations not expressing the markers[31]. Prominin-1 or CD133 is another important marker used for isolating PCSCs. Hermann *et al*[32] demonstrated that CD133+ cells form more tumors than CD133- populations. Another important finding of the study is that cells positive for CD133 and for CXCR4 exhibit a higher metastatic potential than other populations from the same tumors, supporting the observation that CXCR4 may be involved in tumor invasion and metastasis. A recent study provided further evidence for the role of CXCR4 in pancreatic cancer, demonstrating that human pancreatic ductal adenocarcinomas contain a side population of cells with CSC properties and high expression levels of CXCR4 and ABCB1[33]. Moreover, these genes correlate with poor patient survival rates. c-Met is a hepatocyte tyrosine kinase growth factor upregulated by CD44[34]. C-Met was also shown to be a PCSC marker[35,36]. Interestingly, cells expressing c-Met have the same tumor-forming potential as CD44+/CD24+/ESA+. Furthermore, CD133+/c-Met-high are less tumorigenic than CD44+/c-Met-high[35]. Aldehyde dehydrogenase 1 is another marker expressed by cancer stem cells. Studies report that ALDH1 can identify PCSCs and protect the tumor pancreatic cells from programmed cell death induced by radiotherapy[35,37]. Other studies demonstrate that pancreatic cancer stem cells are characterized by genetic and epigenetic alterations associated with carcinogenesis and can form xenograft tumors in immunodeficient mice[38,39].

Limitations of the current methods for isolating cancer stem cells from pancreatic cancer include the lack of specific PCSC markers and the needed to understand the molecular mechanisms that regulate the specific biological properties of PCSCs.

Another important line of research focuses on biomarkers that regulate PCSC properties and behavior[40]. Thus, nestin can modulate important characteristics of PCSCs, such as invasion or metastasis, and may represent a viable target for anticancer therapy. A recent study Lu *et al*[41] reported that Oct 4 and Nanog play important roles in pancreatic cancer by regulating PCSC behavior and suggested that these molecules may represent prognosis markers. Both CD44+/CD24+/ESA+ and pancreatic tumor CD133+ subpopulations are characterized by the overexpression of Nanog, Oct4, Notch1,

MDR1 and ABCG2 and are capable of metastasizing to distant sites, such as the liver[33,42]. Moreover, inhibiting their expression impairs PCSC characteristics. Other reports demonstrate that markers such as DCLK1 can discriminate between normal and tumoral stem cells and that knockdown of DCLK1 decreases molecular pathways that control pancreatic tumorigenesis. Another important regulator of stem-like characteristics in PCSCs is SOX2, which controls cellular proliferation and differentiation[43]. C-kit with KRAS were also proven to modulate the progression of pancreatic adenocarcinoma, supporting the assumption that the use of drugs that downregulate the activity of these markers can improve the prognostic of the disease[44].

One of the main causes of high mortality in this pathology is the resistance to chemotherapy, which is also believed to be mediated by cancer stem cells within the tumor mass[45,46]. In 2013, Lu *et al*[41] demonstrated that in the pancreatic cancer cell line PANC-1, the highly expressed stem cell markers Oct4 and Nanog are associated with chemoresistance, proliferation, migration, invasion, and tumorigenesis *in vitro* and *in vivo*. This study also indicated the potential use of these two transcription factors as prognostic markers and targeted therapies in pancreatic cancer. Another study in a murine model reported that the ALDH+ and CD44+CD24+ cell populations are resistant to treatment with gemcitabine, one of the main chemotherapeutic agents[47].

Shah *et al*[48] has developed a gemcitabine-resistant cell line that exhibits higher expression of the pancreatic CSC markers CD44, CD24, and c-Met, which are also associated with epithelial-mesenchymal transition (EMT).

Aldehyde dehydrogenase (ALDH), considered to be a marker for cancer stem cells, is a detoxification enzyme with increased activity in many cancer types where its presence has been associated with decreased survival[49]. An *in vitro* study revealed that ALDH expression is correlated with the invasiveness of pancreatic cancer cell lines and that patients with ALDH-positive tumors have poor prognosis[49].

It is unclear what the initial molecular events underlying the conversion of tissue stem cells to cancer stem cells in pancreatic cancer; some studies suggest that appearance of c-kit and KRAS mutations might be the primary events in the initial stages of this disease and have proposed c-kit as a potential therapeutic target[44]. Almost all pancreatic cancers are characterized by activating mutations in KRAS and the loss of p16INK4A, but these cancers are also characterized by mutations in the tumor suppressors SMAD4 and p53. More studies suggest the involvement of these genetic alterations in the development of cancer stem cell properties and surface marker profiles.

Another theory suggests that EMT is responsible for the appearance of cells with stem cell-like properties that are characterized by the activation of many pathways involved in EMT[27]. EMT is a crucial process for tumor progression, involving the transformation of epithelial

A

B
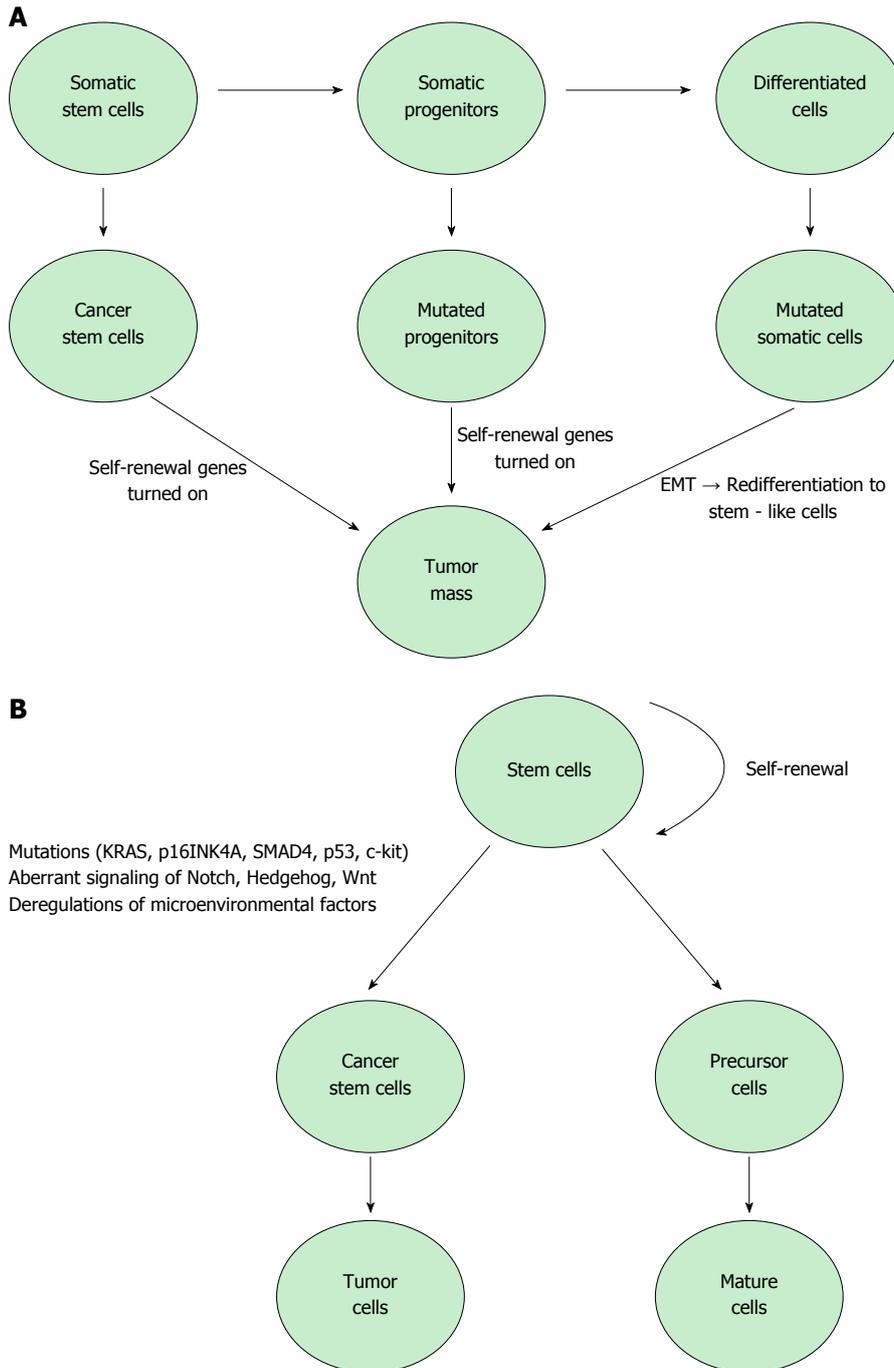
Figure 1 Models that explain tumor development. A: The stochastic model states that every cell has the potential to be the "the cell of origin" of a tumor; B: The "cancer stem cell hypothesis" proposes that tumor evolution is based on stem cells with 'deregulated' signaling pathways. EMT: Epithelial-mesenchymal transition.

characteristics into mesenchymal characteristics, which subsequently allow cancer cells to disseminate from the tumor mass[50].

Signaling pathways involved in pancreatic cancer stem cells

Several signaling pathways are altered in CSCs and EMT-like cells in pancreatic cancer: Hedgehog, Notch, Wnt, AKT and NF-κB (Figure 1). Hedgehog, Notch and Wnt have been shown to be of particular importance in pancreatic cancer stem cells, due to their role in pancreatic

embryonic development and differentiation[51]. These signaling pathways play important roles in the self-renewal of CSCs, tumor growth, invasion, metastasis and resistance to therapy[27]. MiRNAs were recently considered to play a role in the regulation of CSCs[15].

Notch signaling is involved in cell proliferation, survival, apoptosis and the differentiation of pancreatic cells and can promote EMT by controlling some transcription factors and growth factors like Snail, Slug, and TGF-β. Some of the Notch target genes are Akt, cyclin D1, c-myc, COX-2 (cyclooxygenase-2), ERK (extracel-

lular signal-regulated kinase), MMP-9 (matrix metallo-proteinase-9), mTOR (mammalian target of rapamycin), NF-κB (nuclear factor-kappa B), VEGF (vascular endothelial growth factor), p21cip1, p27kip1, and p53, all involved in the development and progression of human cancer. Gemcitabine-resistant pancreatic cancer cells exhibit overexpression of Notch-2 and Jagged-1, whereas Notch1, a key downstream mediator of KRAS, is responsible for pancreatic sphere formation[15,28,52]. Many studies found that pancreatic cancer stem cells resistance to chemotherapy is linked to activated Notch signaling, but the exact mechanism remains unclear[9,53]. There is more evidence detailing that the Notch signaling pathway is essential in supporting the ability of KRAS to transform normal cells into tumor stem cells. In this regard, in pancreatic cancer treatment, Notch signaling inhibition can be more attractive, as long as there are no data arguing that Notch signaling has a critical role in normal adult pancreas homeostasis[54].

Hedgehog signaling is another self-renewal pathway allowing normal stem cells to become independent of control signals; as a result of mutations in this signaling, transformed cells can use Hedgehog for tumor initiation, progression and metastasis. *In vivo* studies revealed that compared with normal pancreatic epithelial cells, CD44+CD24+ESA+ pancreatic cancer stem cells exhibit up-regulation of Shh transcripts, the ligand of Hedgehog signaling[55]. Moreover, 70% of pancreatic cancer tissue exhibits overexpression of Shh, suggesting that Hedgehog signaling may be involved in pancreatic carcinogenesis[15]. Studies in the pancreatic cancer cell line PANC-1 demonstrated that the inhibition of Hedgehog signaling by SMO suppression can reverse EMT, induce apoptosis *via* PI3K/AKT inhibition and inhibit the invasion of pancreatic cancer cells[56]. Moreover, a combination of focal irradiation with Hedgehog signaling inhibition reduces lymph node metastasis in an orthotopic animal model[57].

Wnt/β-catenin signaling is involved in cell proliferation, migration, apoptosis, differentiation, and stem cell self-renewal in several types of cancers[58]. Wnt/β-catenin signaling pathway dysregulation is also associated with chemoresistance in pancreatic cancer, and recent studies suggest that nuclear β-catenin is essential for EMT[50,59]. *In vitro* and *in vivo* studies suggest that activated β-catenin may decrease the differentiation of epidermal stem cells, increase self-renewal capacity and promote epithelial cancers in transgenic mice[60].

In 2013, Sun *et al*[38] reported that one of the most activated signaling pathways in pancreatic cancer stem cells is NF-κB, whose inhibition leads to loss of stem cell properties. This study also indicated that aberrant epigenetic processes, like CpG promoter methylation, can be involved in carcinogenesis mediated by cancer stem cells.

Cancer stem cells from epithelial tissues were identified for the first time in breast cancer in 2003, when Al-Hajj *et al*[61] reported that a distinct population CD44+CD24-/low ESA+ develops tumors in immunodeficient mice.

In pancreatic cancer, the presence of cancer stem cells was reported in 2007 by Shah *et al*[48] who showed that CD44+CD24+ESA+ cells exhibit high tumorigenic potential.

MicroRNAs in pancreatic adenocarcinoma

As often found in many cancers, expression of miRNAs appears to be dysregulated in pancreatic cancer. The miRNA complement of cancer cells appears different than that in normal tissue.

MiRNAs are potent regulators of cell function *via* their role as translational regulators for the synthesis of key proteins. Most often, several miRNAs exhibit different expression profiles in cancer cells.

MiR-21, miR-155 and miR-17-5p appear upregulated in tumoral cells, and these miRs are often called oncogenic miRNAs[62,63]. Similarly, a series of miRNAs, referred to as tumor suppressor miRs miR-34, miR-15a, miR-16-1 and let-7), are downregulated in cancers[64,65].

Key cell differentiation programs during development are controlled by the members of the let-7 and miR-200 families. In cancer, the loss of let-7 leads to disease progression and de-differentiation. The same let-7 family appears as a regulator of EMT and of stem cell maintenance. The EMT process is regulated by miRNA-dependent mechanisms. In human pancreatic cancer, DCLK1 regulates EMT by a mechanism dependent on miR-200a[66,67].

According to Haselmann *et al*[65], the inhibition of the maturation of let-7 by nuclear TRAILR2 in pancreatic cancer cell lines increases their proliferation. This result is consistent with high levels of nuclear TRAIL2 in tissue samples from poor outcome patients.

The population of BxPC-3-LN cells (lymph node metastatic pancreatic cells) contains a 5-fold increased population of CD133+/CXCR4+ cells (stem cell-like cells) compared with the parental (non-metastatic) BxPC-3 cells. Remarkably, a different miRNA pattern is exhibited in CSC-like cells compared with the non-CSC-like cells: up-regulated miR-572, miR-206, miR-449a, miR-489 and miR184 were observed in conjunction with downregulated let-7g-3p, let-7i-3p, let-7a-3p, miR-107, miR-128 and miR-141-5p[68].

The miR-200 family members have been identified as key regulators of cell maintenance and EMT. Tumor progression may represent progressive de-differentiation (EMT) towards a cell type having a stem cell-like phenotype. This process appears to be regulated by miRNA-dependent mechanisms. DCLK1 (a putative marker for pancreatic and intestinal cancer stem cells) regulates EMT in human pancreatic cancer cells *via* a miR-200a-dependent mechanism[69]; DCLK1 also acts as a regulator of let-7a in pancreatic and colorectal cancer cells, supporting the concept that these miRNAs may be novel and relevant targets in solid tumor cancers[63,70]. Sureban *et al*[23] demonstrated that DCLK1 inhibition results in the up-regulation of miRNAs that negatively regulate some key angiogenic and pluripotency factors. In AsPC1

tumor xenografts, the downregulation of c-MYC and KRAS *via* let-7a was observed in a mechanism similar to that demonstrated in pancreatic cancer cells.

The repression of two tumor-suppressor miRs, miR-143 and miR-145, is reported in pancreatic cancer as well in other cancers[71]; moreover, experimental restoration of miR 143/145 levels using nano-vector delivery was demonstrated to inhibit pancreatic cancer cell growth[72]. The miR-143/145 cluster cooperates and inhibits the expression of KRAS2 and RREB1, its downstream effector[71]. MiR-145 was demonstrated to inhibit cell proliferation in lung adenocarcinoma by targeting EGFR. In many cancers, including pancreatic cancer, EGFR is upregulated[73], whereas inhibition of EGF signaling inhibits cancer initiation and progression[74]. Furthermore, a suppressive effect of EGFR on miR-143 and miR-145 was demonstrated in models of colon cancer[75]. These findings are indicators of a negative feedback loop between EGFR and miR-143/145, which is similar to KRAS/RREB1-miR-143/145.

The major role of VEGF signaling *via* its receptors, VEGFR1 and VEGFR2, was demonstrated in tumor vascular growth, angiogenesis, and metastasis, and upregulated angiogenic factors in various cancers (colorectal, breast, renal, liver, and ovarian) have been correlated with poor prognosis. PDAC exhibits endothelial cell proliferation, a mechanisms that increases angiogenesis. Inhibition of VEGF-A, VEGFR1 and VEGFR2 resulted in the inhibition of tumor growth and angiogenesis in mouse models of PDAC. Studies and computational analysis outlined a putative binding site for miR-200 (miR-200a, b and c) in the 3' UTR of VEGFR1 and VEGFR2[76].

More studies suggest that stem cells convert to cancer stem cells by the deregulation of miRNA expression, which affect several signaling pathways involved in proliferation, apoptosis, and more importantly, renewal and differentiation of stem cells[77,78]. Nanog and Sox2, important regulators of stem cell pluripotency, and the CD44 stem cell surface marker are examples of these miRNAs targets[79].

Using microarray analysis, Jung *et al*[70] demonstrated that pancreatic cancer stem cells exhibit differential expression of miR-99a, miR-100, miR-125b, miR-192, and miR-429 compared with controls. An in vitro study conducted on the human pancreatic cancer cell lines AsPC-1, AsPC-1-GTR, MiaPaCa-2, and MiaPaCa-2-GTR revealed re-expression of miRNAs (let-7a, let-7b, miR-26a, miR-101, miR-200b, and miR-200c) that are normally lost in pancreatic cancer and especially in pancreatic spheres can revert or destroy CSCs[80]. Another study reports the loss of miR-34 in CD44+CD133+ MiaPaCa2 pancreatic cancer cells, whereas miR-34 restoration led to the inhibition of a side cell population of tumor cell sphere growth and of tumor formation[64].

Wellner *et al*[71] demonstrated that miR-200c, miR-203 and miR-183 activity can lead to the downregulation of stem cell factors, founding a regulatory feedback loop between miRNAs and CSC in pancreatic cancer.

In this regard, an understanding of miRNAs alterations can lead to the development of better strategies in the treatment of pancreatic cancer patients by the elimination of CSCs.

The identification of dysregulated miR expression and the existence of regulatory loops between miRs and protein regulators of key processes (such as cell growth, angiogenesis, differentiation) suggest the need and potential effectiveness of strategies aiming to restore the "normal phenotype" expression pattern of miRs for cancer treatment. Various approaches have been developed and investigated, such as the delivery of tumor suppressor miRs[81,82], the suppression of expression or action of oncomirs[83,84], targeting the expression of key regulators (such as DCLK1, AMPKα1)[23,85] leading to miR modulation or the simultaneous modulation of multiple miRs, suggesting that using miRs as therapeutic agents or addressing miRNAs as targets represents a potential solution for the therapy of critical cancers.

CANCER STEM CELLS AS THERAPEUTIC TARGETS IN PANCREATIC CANCER

In pancreatic cancer, surgery is usually accompanied by other complementary treatments such as multi-chemotherapy regimens and radiotherapy. Despite clear progress in the detection and treatment of cancer, current strategies fail to completely remove the tumor and prevent recurrence and metastasis. Existing therapies are toxic and nonspecific, being directed towards both normal cells and tumor cells. Most chemotherapeutic regimens are based on gemcitabine but provide a modest improvement in median survival. The response rate was increased by using more than two chemotherapeutic agents[86]. Therapy failure for highly malignant tumors has been explained, at least partially, by the chemo-[87,88] and radioresistant[89] nature of CSCs. Furthermore, studies have demonstrated that gemcitabine regimens, by targeting differentiated cancer cells, lead to a relative enrichment of cancer stem cells[47].

The resistance of CSCs has been explained by several mechanisms: (1) expression of multidrug resistance-linked genes, largely ATP-binding cassette (ABC) drug transporters[90]; (2) activation of Wnt/β-catenin signaling[91]; and (3) activation of Hedgehog pathway[92]. Hence, a series of strategies preferentially target CSCs.

Signaling pathway targeting: Monoclonal antibodies and small molecule kinase inhibitors

TGFβ-related inhibition abrogated the self-renewal capacity of CSCs and *in vivo* tumorigenicity and reversed the resistance of orthotopically engrafted cancer stem cells to gemcitabine. The study demonstrated that the tumor response is, however, limited by the stromal hindering of drug delivery. The addition of a stroma-targeting hedgehog pathway inhibitor enhanced the delivery of the Nodal/Activin inhibitor and translated into long-term, progression-free survival[93].

The *Hedgehog* signaling pathway is usually targeted in experimental designs as an adjuvant to classic chemotherapy. The combined blockade of sonic hedgehog and mTOR signaling together with gemcitabine is capable of eliminating pancreatic CSCs[94]. Inhibition of Smoothened combined with gemcitabine prolonged survival in mice transplanted with pancreatic tumors. Importantly, however, only in mice treated with triple therapy (with mTOR inhibitor rapamycin added) were cancer stem cells virtually completely abrogated, and the authors reported long-term disease stabilization or regression and subsequent long-term survival[95].

Targeting *stemness genes* (*Sox2*, *Oct4* and *c-Myc*) through a complex decoy oligonucleotide designed to simultaneously target all three genes was shown to suppress CSC properties and phenotypes and minimized the tumorigenic capability of the SP cells and the resistance to chemotherapy[42].

Several studies have targeted the *Notch* pathway using selective γ-secretase inhibitors. In pancreatic cancer xenografts, PF-03084014, a selective γ-secretase inhibitor, alone and in combination with gemcitabine, inhibited the cleavage of the nuclear Notch 1 intracellular domain and Notch targets Hes-1 and Hey-1 and induced tumor regression in 3 of 4 subcutaneously implanted xenograft models. The authors argue that the observed effects are due to PF-03084014 targeting putative aggressvive cancer stem cells[54]. Another potent and selective γ-secretase inhibitor, MRK-003, also led to the downregulation of the nuclear Notch1 intracellular domain, the inhibition of anchorage-independent growth, and a reduction in the number of cells capable of extensive self-renewal. Pretreatment of a pancreatic adenocarcinoma cell line with MRK-003 significantly inhibited the subsequent engraftment in immunocompromised mice, and the mixed regimen MRK-003 and gemcitabine in engrafted mice reduced tumor cell proliferation and induced both apoptosis and intratumoral necrosis[96].

Due to their involvement in cell proliferation, receptor tyrosin-kinases are frequently dysregulated in cancers and have been recently therapeutically targeted by small molecule inhibitors. There are reports of pancreatic cancer trials testing both kinase inhibitors and monoclonal antibodies. Sunitinib targets multiple receptor tyrosine kinases, including stem cell factor receptor (c-KIT) and has been shown to possess antitumor efficacy in *in vivo*. The combination of gemcitabine with sunitinib could not surpass the effects of single-agent sunitinib[97]. Cabozantinib, a small kinase inhibitor that targets c-Met and VEGFR2, inhibited viability and spheroid formation and induced apoptosis in pancreatic malignant cells with minor effects in non-malignant cells. In primary, CSC-enriched spheroidal cultures, cabozantinib downregulated the CSC markers SOX2, c-Met and CD133 and induced apoptosis[98].

Tumor-necrosis factor family members have also been targeted as possible anticancer therapies through monoclonal antibodies. A combination of tigatuzumab, a fully humanized death receptor 5 agonist monoclonal antibody, with gemcitabine proved to be more efficacious in killing both CSCs and adenocarcinoma cells. The combination therapy produced a remarkable reduction in pancreatic CSCs, tumor remissions, and significant improvements in the time to tumor progression[99].

Cell cycle regulators represent another class of molecules with the potential to be used as targets in anticancer therapies. Thus, inhibiting checkpoint kinase 1 (Chk1), together with gemcitabine was shown to decrease the capacity of PCSCs to initiate tumors. Another interesting finding was that DNA damage mediated by Chk1 was lower in non-stem cells than in stem cells[100].

Immunotherapy

Given the failure of cytotoxic therapies, new therapy approaches are under investigation. Vaccination therapy aims to increase the patient's immune response against tumor cells by targeting cancer markers with the aid of specialized antigen-presenting cells such as dendritic cells. Currently, there is a number of vaccines for human pancreatic cancer in clinical trials including the following: (1) whole-cell vaccines; (2) combined dendritic cells with antigen to present to patient leukocytes; (3) peptide and DNA vaccines, iv) Ras peptide vaccine; (4) vaccine against common cancer mutations targetable by CD4/8 T cells; (5) telomerase peptide vaccine; (6) CEA and Mucin 1; and (7) survivin-targeted vaccine[101].

Furthermore, boosting the immune response with additional treatment with dendritic cells (LANEX-DC®) was shown to be highly effective and to extend the median survival times up to 8.9 mo[102].

A rather recent and innovative approach in immunotherapy is personalized peptide vaccination (PPV), in which HLA-matched peptides are selected and administered, based on the pre-existing host immunity before vaccination[103]. PPV is now under investigation for pancreatic adenocarcinoma, and a phase Ⅱ study for 41 chemotherapy-resistant advanced pancreatic cancer patients has been reported. Vaccine antigens were selected and administered based on the pre-existing IgG responses to 31 different pooled peptides, and no vaccine-related severe adverse events were observed[104].

Other active agents

Salinomycin is a antiprotozoal agent that was recently proven to preferentially kill breast CSCs[105] and was later investigated in other types of malignancies (reviewed in[106]). In an *in vitro* model of pancreatic adenocarcinoma, salinomycin inhibited the growth of CSCs, and *in vivo* xenografting studies demonstrated that salinomycin combined with gemcitabine could eliminate the engraftment of human pancreatic cancer more effectively than the individual agents[107]. The mechanisms proposed for the anti-tumor activity of salinomycin include the following: (1) inhibition of Wnt/β-catenin signaling[108]; (2) induction of apoptosis and autophagy *via* AMPK activation[108]; (3) increased DNA breakage and phos-

phorylated levels of p53 and H2AX[109]; and (4) cell cycle arrest and apoptosis *via* downregulation or inactivation of cell cycle-associated oncogenes, such as Stat3, cyclin D1, and Skp2[110]. Adamantyl-substituted retinoid-related molecules (ARRs) inhibit growth and induce apoptosis in the pancreatic stem-like cell population, possibly through decreased IGF-1R and β-catenin expression[111].

Isothiocyanate sulforaphane (SF) was used as sensitizer of pancreatic CSCs to TRAIL (tumor necrosis factor-related apoptosis inducing ligand)-induced apoptosis by quercetin and sorafenib. Combination of SF with a cytotoxic drug efficiently induced apoptosis along with the inhibition of self-renewing potential, ALDH1 activity, clonogenicity, xenograft growth and relapse of GEM-treated tumor cells in nude mice[112].

The flavonoid Quercetin enhances TRAIL-mediated apoptosis, acts as a chemosensitizer for the ABC pump-proteins and can enhance the effects of sulforaphane in inhibiting pancreatic CSC characteristics[113].

Delivery of cytotoxic drugs by specific targeting of stem cell markers

Targeted therapeutic delivery is a way to ensure that drugs reach the designated target at the highest concentration within safety limits. Targeted delivery relies on nanoparticles [small metallic or non-metallic molecules, (such as polymeric, carbonic, sillica-for a detailed review please see[114])]. Most nanoparticles accumulate in tumors due to their intense and leaky neovascularization, but some can be retained in the tumors with the use of cancer-specific antigens[115]. In the same manner that nanoparticles are targeted for the bulk tumor, nanoparticles can be targeted for CSCs by CD-133, for example. To increase delivery into the cytosol and prevent early lysosomal degradation, Bostad *et al*[116] have employed photochemical internalization (PCI), a minimally invasive method for light-controlled, specific delivery of membrane-impermeable macromolecules to increase the cytotoxic effect of an immunotoxin targeting CD133-expressing cancer cells of colon (WiDr and HCT116) and pancreas (BxPC-3) origin.

CONCLUSION

Pancreatic cancer remains one of the major causes of cancer death with low survival rates due to the metastasis of early-stage tumors and the lack of any effective treatment. Discoveries made in recent years clearly demonstrate that stem cells and EMT-type cells are involved in pancreatic cancer and are responsible for chemoresistance and the metastatic potential of this tumor type. The emergence of cancer stem cells is based on genetic alterations and modifications in signaling pathways that result in the transformation of normal stem cells, progenitors or differentiated cells. Currently, cancer stem cell inhibitors in combination with conventional therapy are being tested in clinical trials and could provide an innovative approach for the treatment of pancreatic cancer.

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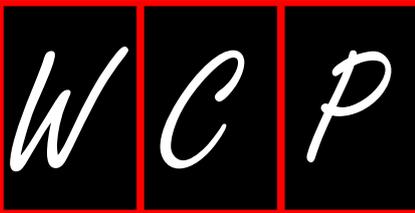
Prognostic factors related with survival in patients with pancreatic adenocarcinoma

Ahmet Bilici

Ahmet Bilici, Department of Medical Oncology, Medical Faculty, Istanbul Medipol University, 34214 Bagcilar, Istanbul, Turkey

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Correspondence to: Ahmet Bilici, MD, Department of Medical Oncology, Medical Faculty, Istanbul Medipol University, TEM Avrupa Otoyolu Goztepe Cikisi No: 1, 34214 Bagcilar, Istanbul, Turkey. ahmetknower@yahoo.com

Telephone: +90-532-5280486 Fax:+90-216-4422947

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Abstract

The prognosis in patients with pancreatic cancer is poor and this cancer is the fourth leading cause of cancer-related death worldwide. Although surgical resection is the only curative treatment of choice for pancreatic cancer, the majority of patients are diagnosed at an advanced stage, thus only 10%-15% of them are suitable for curative resection and the overall survival is less than 5%. Chemotherapy for metastatic disease is to palliate symptoms of patients and to improve survival. Therefore, prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer. This article reviews the prognostic factors affecting survival of patients with pancreatic cancer in the light of recent advances in the literature.

Key words: Pancreatic cancer; Prognostic factors; Survival; Carbohydrate antigen 19-9; Treatment

Core tip: The overall prognosis associated with pancreatic cancer has not improved over the last 20 years, even if new diagnostic and therapeutic strategies have emerged. Thus, investigations on predictive factors in pancreatic cancer are needed because these factors should have predictive value in relation to longer survival after surgery than after palliative treatment. Prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer.

INTRODUCTION

Pancreatic adenocarcinoma still remains a major public health issue and is the fourth leading cause of cancer-related death worldwide[1]. Although surgical resection is the only curative treatment of choice for pancreatic cancer, unfortunately, the majority of patients are diagnosed at an advanced stage, and thus only 10%-15% of them are suitable for curative resection and the overall survival is less than 5%[2,3]. Chemotherapy is used in the adjuvant setting and in the treatment of locally advanced inoperable and metastatic disease.

The primary goals of chemotherapy for metastatic disease are palliation and improved survival[4,5]. Therefore, identifying poor prognostic factors that may predict the tumor recurrence and prognosis of patients is important for selecting appropriate treatment protocols. So it is important to determine new biological or pathological indicators related to survival in addition to well-

known prognostic factors such as clinical and pathological stage, performance status, and surgical margin[6]. In this article, the prognostic factors affecting survival of patients with pancreatic cancer were reviewed.

SURGICAL AND PATHOLOGICAL FACTORS

The primary surgical or pathological factors that influence prognosis are whether the tumor is localized at the pancreas and whether the tumor has spread to lymph nodes or distant organs[1] because the highest cure rate occurs if the tumor is truly localized to the pancreas. In the present TNM staging system, tumor size, peripancreatic extension, and vascular involvement are used. Traditionally, TNM staging, especially in the presence of metastasis (advanced stage), has been found to be an important prognostic factor in patients with pancreatic cancer for survival[7-9].

Surgical margin

Surgical resection is the only potentially curative option for treatment of pancreatic cancer and the nature of surgery for resectable tumors depends on the tumor localization and size. The incidence of R1 resection has been indicated as being 20% in the literature, but the improvement of pathological work-up procedures has increased the rate of R1 resection up to 80%[10,11]. Menon et al[12] reported that of 27 patients with pancreatic cancer, 22 patients underwent R1 resection and the median survival rate for patients with R1 resection was significantly worse than that of patients with R0 resection (14 mo vs not reached). In a study performed by Raut et al[13], they reported that the rate of R1 resection was 16.7% and patients who underwent an R1 resection had a median overall survival (OS) of 21.5 mo compared with 27.8 mo in patients who underwent an R0 resection. In addition, multivariate analysis showed that high mean operative blood loss and large tumor size were independent predictors of an R1 resection, but margin status did not independently influence survival.

Another study including 265 pancreatic carcinoma patients who had undergone surgical resection reported that R1 resection in 49 patients (51%) and R2 resection in four patients (4%) were performed[14]. The R1-positive margin was localized at the retroperitoneal resection margin in 76% and at the trans-section margin in 14% of tumors. Median survival time was better in R0-resected patients compared with R1-resected patients (22 mo vs 15 mo). A positive resection margin after pancreatic resection is considered to be a poor prognostic factor, and some have proposed that an R1 margin may be a biologic predictor of more aggressive disease. On the other hand, whether these patients with pancreatic carcinoma who underwent margin-positive resection have to be managed with aggressive treatment modalities has not been described.

Lymph nodes status and lymph node ratio

Lymph node ratio (LNR) may be more useful than nodal (N) status in prognostic subclassification of pancreatic adenocarcinomas after pancreatoduodenectomy. Recent studies have suggested that LNR may also be an important prognostic factor in pancreatic cancer[15-17]. In the TNM staging system, the number of resected lymph nodes may be very important, but node-positive patients are not a homogenous group, because stage migration may occur in resected pancreatic cancer patients. To resolve these limitations, recently LNR was proposed as a new prognostic factor by several authors to prevent the 'stage migration' phenomenon[15-17]. Riediger et al[17], in 204 resected patients, reported that LNR was the strongest predictor of survival and they concluded that the routine estimation of the LNR may be helpful not only for the individual prediction of prognosis but also for the indication of adjuvant therapy. The analysis of Surveillance, Epidemiology, and End Results and MGH (Massachusetts General Hospital) in 10254 and 827 resected patients, respectively, showed that higher LNR (> 0.2) was associated with worse survival by univariate analysis, and in addition the hazard ratio (HR) raised proportionally when more lymph nodes were examined in multivariate analysis. This study concluded that while the contribution of the number of positive nodes to survival was relatively small, LNR was strongly associated with survival, and thus, LNR provided a stronger and more accurate predictor of survival than the number of positive nodes[18].

Perineural and blood vessel invasion

Both perineural (PNI) and blood vessel invasion (BVI) have been previously investigated in patients with pancreatic cancer and found to be important prognostic indicators for survival[14,19,20]. Lee et al[19] showed that PNI was an important adverse prognostic factor for patients with surgical resection, as was pN stage. In a study performed by Chatterjee et al[21], PNI and BVI were found to be associated with the OS and lymph node status in patients who were treated with neoadjuvant treatment. The median OS for patients with PNI was worse than that of patients without PNI (22 mo vs 36 mo). Moreover, the median OS was better in patients without BVI compared with patients with BVI (34 mo vs 22 mo). They detected that retroperitoneal resection margin was correlated with the presence of both BVI and PNI. The authors concluded that PNI and BVI were significantly poor prognostic indicators.

Tumor localization

Some studies have investigated the prognostic significance of tumor localization in pancreatic cancer patients, but there is currently no consensus[7-9,19,22]. In a study performed by Park et al[8], univariate analysis indicated that tumor location was an important prognostic factor for

Table 1 Surgical and pathological factors in pancreatic cancer

Ref.	No. of patients	Results
Surgical margin/resection (R1 vs R0)		
Menon et al[12]	27	mOS, 14 mo vs NR
Raut et al[13]	360	mOS, 21.5 mo vs 27.8 mo
Lymph nodes status and lymph node ratio		
Riediger et al[17]	204	LNR was an independent prognostic factor
Valsangkar et al[18]	14907	LNR was strongly correlated with survival
Perineural and blood vessel invasion		
Chatterjee et al[21]	86	mOS, 34 mo for BVI (-) vs 22 mo for BVI (+); mOS, 32 mo for PNI (-) vs 22 mo for PNI (+)
Tumor localization		
Park et al[8]	340	It was an important prognostic factor by univariate analysis
Zhang et al[7]	302	It was an independent prognostic indicator
Operative factors		
Nagai et al[23]	271	OBL greater than 2000 mL was an independent prognostic factor for OS
Keck et al[24]	270	PBT was an independent prognostic indicator for survival

mOS: Median overall survival; NR: Not reach; LNR: Lymph node ratio; BVI: Blood vessel invasion; PNI: Perineural invasion; OBL: Operative blood loss; PBT; Perioperative blood transfusion.

OS, but the significance of tumor site as an independent prognostic indicator could not be proved in the multivariate analysis. Lee et al[22] showed that high CEA level was significantly correlated with tumor location. In the patients with elevated CEA level, tumors were located mostly at the pancreas body and tail. The authors could not show that tumor location was a prognostic factor by multivariate analysis, although in the univariate analysis it was detected as being a prognostic factor. However, in another study carried out by Zhang et al[7], localization of the primary tumor was found to be an independent prognostic factor. In other words, the mortality risk was increased for tumors located at the body and tail of the pancreas compared to the tumors located at the head and neck of the pancreas.

Operative factors
An influence of operative blood loss (OBL) on survival in patients with pancreatic cancer after curative resection has been investigated. Nagai et al[23] retrospectively analyzed 271 patients and found that the OS was significantly affected by the amount of OBL. The median survival times were 26.0, 15.3, and 8.7 mo for OBL less than 1000, 1000 to 2000, and greater than 2000 mL, respectively (< 1000 mL vs 1000-2000 mL, $P = 0.019$; 1000-2000 mL vs > 2000 mL, $P < 0.0001$). Moreover, OBL greater than 2000 mL was also detected to be an independent prognostic factor in multivariate analysis

(HR = 2.55) and OBL of 2010 mL was found to be an appropriate cut-off level to predict early mortality within 6 mo after resection. Male sex, year of resection, and plexus invasion were independently associated with OBL greater than 2000 mL. In light of these results, the authors concluded that excessive OBL was found to be a prognostic determinant of survival and it can be used to stratify the risk for pancreatic cancer mortality after surgery for pancreatic cancer. On the other hand, prognostic significance of perioperative blood transfusion (PBT) has also been reported. In a study performed by Keck et al[24], PBTs were given in 46% of 270 pancreatic cancer patients. Univariate analysis showed that PBT was related with poorer survival, as were positive margins, more than one involved node, and poorer grading. In addition, they found that PBT was an independent prognostic indicator for survival by multivariate analysis after resection. The authors thought that impact of PBT was independent of the perioperative complications or resection type. Table 1 shows selected trials of surgical and pathological prognostic factors in pancreatic cancer.

CLINICAL FACTORS

Performance status
Some studies have evaluated the impact of performance status (PS) on survival for patients with pancreatic adenocarcinoma, but the results are conflicting. In a study carried out by Sezgin et al[25], the authors reported that only PS was an independent prognostic factor for OS in patients with advanced pancreatic cancer. Similarly, Tas et al[26] found that initial poor PS (PS 2-4) was significantly associated with worse survival for patients with all stages of pancreatic cancer. In addition, poor PS remained as an independent prognostic indicator for survival by multivariate analysis and in patients with poor PS, severe weight loss (> 10%), large tumor diameter (> 3 cm), and especially metastatic disease was related with significantly shorter OS. On the other hand, in another study, although an influence of PS on survival was detected in the univariate analysis, its prognostic significance was lost in multivariate analysis[8]. Lee et al[22] showed that in the elevated CA19-9 level group (≥ 37 U/mL), PS was significantly higher compared with the normal CA19-9 group. Furthermore, PS (0 vs 1-2) was found to be an important prognostic factor in the univariate analysis for OS.

Diabetes mellitus, obesity and jaundice
Diabetes mellitus (DM) is commonly diagnosed in pancreatic cancer patients, but the significance of new-onset DM as a cause of underlying pancreatic cancer is unknown. Some studies have investigated the prognostic significance of DM in pancreatic cancer[8,25,27], but an impact of DM on survival could not be proved.

Cachexia is a known characteristic of pancreatic cancer with detects as 80% of patients cachexic at diagnosis. Therefore, measurement of body mass index (BMI) at

the time of diagnosis does not provide accurate representation of a patient's long-term exposure to obesity[28]. However, some studies have shown that high BMI is associated with increased risk of pancreatic cancer incidence and mortality[29,30]. On the other hand, studies of obesity and survival in patients with pancreatic cancer are notably controversial. In a population-based study including 510 patients with pancreatic cancer, Gong et al[31] indicated that elevated HR of 1.3 was detected for obese (BMI ≥ 30) compared with normal range BMI (< 25) patients. But, the relation between OS and BMI could not be found. Similarly, recent study evaluated the association of BMI with the risk of death from pancreatic cancer in a pooled analysis of data from Asia Cohort Consortium[32]. It did not support an relation between BMI and risk of death from pancreatic cancer. As a different these studies, in a study carried out by Yuan et al[33] the association of prediagnostic BMI with pancreatic cancer survival was analyzed. Higher prediagnostic BMI was associated with more advanced stage at diagnosis, with 72.5% of obese patients presenting with metastatic disease versus 59.4% of healthy-weight patients. Furthermore, higher baseline BMI was associated with reduced survival. HR for death was 1.53, comparing BMI ≥ 35 kg/m^2 with BMI < 25 kg/m^2 ($P = 0.001$).

In a study performed by Smith et al[34], the presence of preoperative jaundice was found to be associated with poor survival in patients with pancreatic cancer. Another study showed that preoperative jaundice was the only independent prognostic factor for pancreatic cancer patients[19]. On the other hand, Perini et al[35] demonstrated that both preoperative DM and jaundice had no adverse effect on survival for curative resection in pancreatic cancer patients. Recently, Strasberg et al[36] analyzed 400 patients with resected pancreatic cancer, and preoperative jaundice was found to be a significant indicator of poor outcome in the multivariate analysis. Moreover, the relationship was detected between jaundice and nodal status, and jaundiced patients who underwent preoperative stenting had a survival advantage. The underlying mechanism related with the influence of jaundice on survival is unknown and additional studies are required to determine the exact mechanism for this effect.

Treatment and gemcitabine

Chemotherapy is only modestly effective in advanced disease but has a significant impact in the adjuvant setting, with 5-fluorouracil and gemcitabine both having efficacy in a subgroup of patients and increasing 5-year survival from 10%-15% with surgery alone to 20%-25%[37-40]. Park et al[8] analyzed 340 patients with pancreatic cancer and of 141 stage Ⅲ patients, 57 received supportive care (BSC) only, 25 received chemotherapy (CT), and 59 received concurrent chemoradiotherapy (CCRT); of the 199 stage Ⅳ patients, 119 were treated with BSC only and 80 received CT. Univariate analysis showed that CT and CCRT were significant prognostic indicators for OS in stage Ⅲ patients compared with patients that received

BSC only (11.3 mo vs 10.4 mo vs 6.4 mo, respectively; $P < 0.001$). Similarly, in stage Ⅳ patients, median OS for patients who were treated with CT was significantly better than that of patients who received BSC only (6.4 mo vs 3.1 mo, $P < 0.001$). In addition, initial treatment effect remained an independent prognostic factor compared to BSC only in the multivariate analysis[8].

In a study performed by Lee et al[19], gemcitabine chemotherapy was found to be the only independent prognostic indicator for OS in advanced or unresectable pancreatic cancer patients who had undergone palliative surgical by pass. Moreover, Zhang et al[7] evaluated 302 all-stage pancreatic cancer and found that the median OS of patients who did not receive any treatment or those treated with BSC only was 1.3 mo, while the median OS for patients who had undergone surgery, CT, biliary drainage therapy, arterial interventional CT, and comprehensive CT was 11.0, 7.3, 3.5, 9.0, and 11.0 mo, respectively ($P < 0.05$). In the multivariate analysis, the presence of treatment vs no therapy or BSC only was an independent prognostic factor (HR = 13.93, $P = 0.000$). However, platinum combination CT was significantly associated with improved OS compared to non-platinum CT regimen (HR = 0.56, $P = 0.011$). Selected trials related with clinical prognostic factors are summarized in Table 2.

LABORATORY AND MOLECULAR FACTORS

Prognostic role of carbohydrate antigen 19-9 levels

Serum carbohydrate antigen (CA) 19-9, the sialylated Lewis blood group antigen defined by the monoclonal antibody 1116 NS 19-9, is a tumor-associated antigen synthesized by normal pancreatic and ductal cells[41]. CA19-9 is considered to be the standard serum marker of pancreatic cancer due to its high sensitivity of 70%-90% and specificity of around 90%[42]. Serum CA19-9 levels have been found to be a useful tumor marker in differentiating benign from malignant pancreatic lesions, and to monitor tumor response to treatment[42,43]. Previous studies suggested that preoperative CA19-9 levels could predict the resectability of pancreatic cancer[44,45], and other studies reported that pretreatment CA19-9 level was an important prognostic factor in patients with pancreatic cancer who received CT or CCRT[8,9,45,46].

Park et al[8] reported that elevated CA19-9 levels (> 670 U/mL) were found to have prognostic significance for OS by univariate analysis, while it was an independent prognostic factor for OS in the multivariate analysis. Furthermore, another study found similar findings. The median OS time for patients with high CA19-9 level was worse than that of patients with normal CA19-9 level (3.8 vs 5.0 mo), which was not significant, but multivariate analysis indicated that it was an independent prognostic indicator for OS (HR = 4.54, $P = 0.033$)[7]. Recently, in a study by Humphris et al[47], low postoperative CA19-9 at 3 mo and before adjuvant chemotherapy were indepen-

Table 2 Clinical prognostic factors in pancreatic cancer in selected trials

Ref.	No. of patients	Results
Performance status		
Sezgin et al[25]	67	PS was an independent prognostic factor for OS
Tas et al[26]	335	Initial poor PS (2-4) was significantly associated with worse survival
DM, obesity and jaundice		
Gong et al[31]	510	HR = 1.3 for patients with BMI ≥ 30 compare to those with BMI < 25. But no correlation was found between BMI and survival
Yuan et al[33]	902	Higher baseline BMI was associated with reduced survival
Smith et al[34]	155	The presence of jaundice at the time of surgery was a significant adverse predictor of early survival
Strasberg et al[36]	400	The preoperative jaundice was found to be a significant indicator of poor outcome
Treatment		
Park et al[8]	340	mOS, 11.3 vs 10.4 vs 6.4 mo for stage III patients treated with CT, CCRT and BSC, respectively ($P < 0.001$)
		mOS, 6.4 vs 3.1 mo for patients with stage IV treated with CT or BSC, respectively ($P < 0.001$)
Lee et al[19]	82	Gemcitabine chemotherapy was found to be the only independent prognostic indicator for OS in advanced pancreatic cancer

DM: Diabetes mellitus; mOS: Median overall survival; PS: Performance status, BMI: Body mass index; CT: Chemotherapy; CCRT: Concurrent chemoradiotherapy; BSC: Best supportive care.

dent prognostic factors (median OS; 25.6 mo vs 14.8 mo, $P = 0.0052$) in 260 patients with pancreatic cancer who underwent surgical resection. Patients with postoperative CA19-9 levels > 90 U/mL did not benefit from adjuvant chemotherapy compared with those with a CA19-9 level of ≤ 90 U/mL (median OS 26.0 mo vs 16.7 mo, $P = 0.0108$). Normalization of CA19-9 within 6 mo of resection was also an independent favorable prognostic factor (median OS: 29.9 mo vs 14.8 mo, $P = 0.0004$) and normal perioperative CA19-9 levels were identified as being a good prognostic group, which was associated with a 5-year survival of 42%.

Other tumor markers

Carcinoembryonic antigen (CEA) is the standard tumor marker and is commonly used for predicting treatment response and prognosis of patients with colorectal cancer[48]. In contrast to the CA19-9 level, an impact of CEA on survival of pancreatic cancer patients has not yet been determined, but CEA might be beneficial in predicting pancreatic cancer. Zhang et al[7] in their study including 302 patients with pancreatic cancer reported that the patients with high CEA levels had a median survival of 2.0 mo compared to patients with normal levels (5.0 mo). This difference was statistically significant (HR = 1.43, $P = 0.030$). However, the significance of CEA levels as an independent prognostic factor could not be proved in the multivariate analysis. In a study carried out by Lee et al[22], they retrospectively analyzed 187 pancreatic cancer patients, and reported that the median OS time for patients with normal CEA levels was significantly better than that of patients with high CEA levels (16.3 mo vs 10.2 mo, $P = 0.004$). In addition, elevated CEA levels were found to be an independent prognostic factor in the multivariate analysis.

Despite these findings, to detect whether CEA can be applicable as a prognostic marker of pancreatic cancer, it should be evaluated in a large number of patients with all stages of pancreatic cancer. Various tumor mark-

ers such as CA125, CA15-3, CA72-4, and CA242 have also been analyzed, but their importance as independent prognostic indicators could not be definitively demonstrated[7,49].

Hematological parameters

Platelet, lymphocyte, and neutrophil counts, mean platelet volume, and the ratios of various hematologic cells have been shown to be valuable prognostic factors in various malignancies, such as renal, gynecological, and colorectal cancers[50-53]. Schwarz et al[54] demonstrated that preoperative platelet count predicts survival after resection of pancreatic adenocarcinoma. On the other hand, in a study comprising 205 patients performed by Domínguez et al[55], there was no evidence to support preoperative platelet count as either an adverse or favorable prognostic factor in pancreatic ductal adenocarcinoma, which was not compatible with a study of Zhang et al[7]. Despite conflicting results regarding platelet counts, white blood cells (WBCs) were found to be an independent prognostic factor for OS in patients with pancreatic cancer in two studies[7,46]. Although low hemoglobin levels were associated with poorer OS time, the significance as an independent prognostic marker could not be proved by the multivariate analysis[7].

The prognostic value of pretreatment platelet to lymphocyte ratio (PLR) and neutrophil to lymphocyte ratio (NLR) in patients with pancreatic cancer has also been evaluated[56,57]. Preoperative PLR has been defined as an independent significant prognostic marker by Smith et al[58] in resected pancreatic ductal adenocarcinoma. In the same study, the median overall survival in patients with a PLR of 150 or less was 19.7 mo, 13.7 mo in those with a PLR of 151-300, and 5.8 mo in patients with a value of > 300. Aliustaoglu et al[57] showed that there was no statistically significant difference between cases with PLR values ≤ 160 and > 160. However, they analyzed NLR in the same patients with pancreatic cancer. Patients with a NLR value of < 5 had a significantly higher median

Table 3 Selected trials of laboratory prognostic factors in pancreatic cancer

Ref.	No. of patients	Results
CA 19-9 levels		
Park et al[8]	340	Elevated CA19-9 levels (> 670 U/mL) were found to independent prognostic factor for OS
Zhang et al[7]	302	mOS, 3.8 mo for patients with high CA 19-9 levels vs 5.0 mo for those with normal CA 19-9 levels
Humphris et al[47]	260	mOS, 25.6 mo for low postoperative CA 19-9 levels vs 14.8 mo for high CA 19-9 levels
		Normalization of CA19-9 within 6 mo of resection was also an independent favorable prognostic factor
Other tumor markers		
Zhang et al[7]	302	mOS, 2.0 mo for patients with high CEA levels vs 5.0 mo for those with normal CEA levels
Lee et al[22]	187	mOS was 16.3 and 10.2 mo for patients with normal CEA vs high CEA levels, repsectively
Hematological factors		
Zhang et al[7]	302	WBCs were independent prognostic factor for OS
Smith et al[58]	110	mOS in patients with a preoperative PLR of 150 or less was 19.7 mo, 13.7 mo in those with a PLR of 151-300, and 5.8 mo in patients with a value of > 300
Aliustaoglu et al[57]	65	Patients with a NLR value of < 5 had a significantly higher median OS time compared to those with a NLR value of ⩾ 5
Stotz et al[56]	371	An increased NLR as an independent prognostic factor for inoperable and surgically resected patients
Biochemical parameters		
Zhang et al[7]	302	Serum albumin and BUN levels were found to be independent prognostic factors for prediction of OS
Stocken et al[46]	653	Albumin, ALP, LDH, BUN, and AST were independent prognostic indicators for survival of advanced pancreatic cancer
Haas et al[60]	291	Pretreatment LDH levels were significantly associated with TTP. Baseline LDH, CRP, and bilirubin were significant prognostic factors for OS

mOS: Median overall survival; WBC: White blood cell; PLR: Platelet to lymphocyte ratio; NLR: Neutrophil to lymphocyte ratio; BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TTP: Time to progression; CRP: C-reactive protein; CEA: Carcinoembryonic antigen.

OS time compared to those with a NLR value of ⩾ 5 ($P = 0.015$). Recently, Stotz et al[56] evaluated NLR in 371 patients with primary operable and inoperable pancreatic cancer. They reported that multivariate analysis identified increased NLR as an independent prognostic factor for inoperable PC patients (HR = 2.53, $P < 0.001$) and surgically resected pancreatic cancer patients (HR = 1.61, $P = 0.039$). Furthermore, in inoperable pancreatic cancer patients, the modified Glasgow prognostic score was associated with poor cancer-specific survival only in univariate analysis (HR = 1.44). In light of these findings, the authors concluded that risk prediction for cancer-related end points using NLR does add independent prognostic information to other well-established prognostic factors in patients with pancreatic cancer, regardless of the undergoing therapeutic modality. Thus, the NLR should be considered for future individual risk assessment in pancreatic cancer patients.

Biochemical parameters

Some serum chemistry markers such as albumin, lactate dehydrogenase (LDH), bilirubin, creatinine, and blood urea nitrogen (BUN) have previously been tested, but the prognostic role of these markers has not yet been fully defined. Serum albumin and BUN levels were found to be independent prognostic factors for prediction of survival in pancreatic cancer, while total bilirubin, direct bilirubin, glutamic-pyruvic transaminase, glutamic-oxalacetic transaminase, serum creatinine, and LDH were not[7]. However, the patients with high serum LDH levels had poor prognosis compared to those with normal levels (4.3 mo vs 7.0 mo) by univariate analysis. Tas et al[59] demonstrated that high serum LDH levels

were significantly associated with tumor burden and reflected tumor growth and invasion potential in patients with pancreatic cancer. Similarly, Stocken et al[46], in their study including 653 pancreatic cancer patients, detected that albumin, alkaline phosphatase (ALP), LDH, BUN, and aspartate aminotransferase (AST) were independent prognostic indicators for survival in patients with advanced pancreatic cancer. A recent study conducted by Haas et al[60] showed that in univariate analysis, pretreatment LDH (HR = 2.04) levels were significantly associated with time-to progression (TTP). Regarding OS, baseline LDH (HR = 2.07), C-reactive protein (CRP) (HR = 1.69), and bilirubin (HR = 1.62) were significant prognostic factors. In the multivariate analyses, pre-treatment bilirubin and CRP for OS had an independent prognostic value. They concluded that CRP, LDH, and bilirubin can also provide prognostic information on TTP and OS. Table 3 indicates selected trials of laboratory factors in pancreatic cancer.

Molecular markers

Gemcitabine is transported into the cell mainly by human equilibrative nucleoside transporter 1 (hENT1) (also known as SLC29A1). hENT1 has been investigated as a predictive biomarker of gemcitabine efficacy, mostly in pancreatic cancer, and populations of cells with lower hENT1 expression may be relatively gemcitabine resistant due to reduced intracellular accumulation of the drug[61]. Previous studies suggest that hENT1 protein expression is associated with increased OS and DFS in pancreatic cancer patients who received gemcitabine[62,63]. Recently, in patients who were included in the ESPAC 1-3 trials and were treated with adjuvant gemcitabine or

5-fluorouracil (5-FU), the results of tissue microarrays for hENT1 was presented at the 2013 ASCO annual meeting[64]. The median OS time for patients with high hENT1 expression who received gemcitabine was significantly better than that of patients with low hENT1 expression (26.2 mo *vs* 17.1 mo, $P = 0.002$). However, there was no difference among patients treated with 5-FU with respect to hENT1 expression. The authors concluded that patients with high hENT1 expression might benefit more from gemcitabine treatment.

SPARC (secreted protein and rich in cysteine), a matricellular protein found to be under-expressed in certain cancers, has emerged as a multifunctional protein capable of inhibiting the growth of pancreatic, colorectal, and ovarian cancers[65,66]. The significance of expression of SPARC as a prognostic factor in the stroma of pancreatic tumors has been shown[67]. In a study performed by Sinn *et al*[68], immunohistochemistry in the tissue sample for expression of SPARC in the stroma around the tumor, but also in the tumor cell, of patients from the Charité Onkologie (CONKO)-001 study was carried out and their results were presented at the 2013 ASCO annual meeting. Patients who received gemcitabine as adjuvant treatment had a longer DFS and OS when stromal and cytoplasmic expression of SPARC was not-strong or negative, respectively, compared with strong expression of SPARC. Thus, SPARC expression estimation, both in the tumor or its stroma, seems to be a valuable prognostic factor in patients receiving gemcitabine as adjuvant therapy in patients with pancreatic cancer.

The prognostic significance of circulating tumor cells (CTCs) has been investigated and patients who had CTCs (more than 1 in 7.5 mL) before curative surgery, or after therapy initiation, has a trend towards poorer OS or PFS[69]. Bidard *et al*[70] prospectively analyzed patients with locally advanced unresectable pancreatic cancer before and after 2 mo of chemotherapy for CTCs. More than one tumor cell in 7.5 mL was considered as positive. Before treatment, 5% of patients had positive detection of CTCs and 9% at the end of 2 mo of therapy. This positivity was found to be associated with poor tumor differentiation and the OS was shorter in these positive patients. The determination of CTCs in patients with pancreatic cancer seems to have a negative prognostic role[71]. There is a significant relationship between the amount of peritumoral CD4+ and CD8+ T-cells and survival in patients with pancreatic cancer and it was found to be an independent prognostic factor for OS[71].

Transforming growth factor β (TGF-β) acts as suppressor and promoter of cancer progression. Intracellular Smad proteins (common mediator SMAD4) play a pivotal role in mediating antimitogenic and proapoptotic effects of TGF-β[72]. In 55% of pancreatic tumors SMAD4 alterations are found and *it* is inactivated in the majority of pancreatic adenocarcinoma with concurrent mutational inactivation of the *INK4A/ARF* tumor suppressor locus and activation of the *KRAS* oncogene[73]. Previous reports revealed unclear results related with SMAD4 as a predictor of survival in pancreatic cancer[74-76]. Blackford *et al*[76] reported that SMAD4 gene inactivation was associated with poorer prognosis in resected pancreatic adenocarcinoma. In other words, median survival time in patients without SMAD4 gene inactivation was significantly better than those with inactivation (14.2 mo *vs* 11.5 mo, $P = 0.006$). Recent study showed a significant relationship was found between SMAD4 expression and tumor size ($P = 0.006$), lymphatic invasion ($P = 0.033$), and lymph node metastasis ($P = 0.006$)[77]. Moreover, loss of SMAD4 expression was significantly associated with shorter OS and it was found to be an independent prognostic factor for both OS and DFS by multivariate analysis. Similarly, another study has confirmed these results[78].

Novel prognostic biomarkers

Hypoxia-inducible factor 1 alpha (HIF1α) has been found to be an unfavorable prognostic indicator in many cancers and is known to regulate some genes in the angiogenesis pathway[79]. Some studies have previously been showed that HIF1α had a strong impact on the prognosis of patients with pancreatic adenocarcinoma[80-82]. NEDD9, a focal adhesion scaffolding protein, has been recently proposed to regulate invasion and metastasis in some cancer types[83-85]. In a study performed by Xue *et al*[86], they investigated the expression and prognostic significance of NEDD9 in patients with pancreatic cancer. NEDD9 protein and mRNA levels were elevated in pancreatic carcinoma lesions compared with noncancerous tissues. A high NEDD9 expression level was significantly correlated with clinical staging, lymph node metastasis, and histological differentiation. The median survival time for patients with a higher NEDD9 expression was significantly shorter than that of patients with lower NEDD9 expression. In addition, the multivariate analysis revealed that NEDD9 was an independent factor of poor prognosis.

FOXM1 (Forkhead box M1) is a typical proliferation-related transcription factor and is also intimately involved in tumorigenesis. It induces cell proliferation and cell cycle progression by promoting the entry into S-phase and M-phase[87]. Xia *et al*[88] in their study, evaluated correlation between FoxM1 expression level and survival of patients with pancreatic adenocarcinoma. They showed that a high level of expression of FoxM1 was significantly correlated with clinical staging, lymph node metastasis, and histological differentiation. Furthermore, patients with a higher FoxM1 expression had a significantly shorter survival time compared to patients with lower FoxM1 expression and FoxM1 was found to be an independent factor for survival.

Recent study indicated that B7H4, HSP27 and DJ-1 protein expressions in the tissue specimens of 41 patients with resected pancreatic cancer were independently associated with a negative impact of chemotherapy with gemcitabine on patient's survival[89]. In addition, patients who overexpressed B7H4 had worse prognosis than patients without overexpression. In a study carried

Table 4 Molecular and novel biomarkers as prognostic factors in pancreatic cancer

References	No. of patients	Results
Molecular markers		
Neoptolemos et al[64]	48	mOS, 26.2 mo for patients with high hENT1 expression *vs* 17.1 for those with low hENT1 expression who treated with gemcitabine (*P* = 0.002)
Sinn et al[68]	160	Strong stromal SPARC expression was associated with worse DFS and OS (strong *vs* not-strong DFS 9.0 *vs* 12.6 mo, *P* = 0.005; OS 19.8 *vs* 26.6 mo (*P* = 0.033).Cytoplasmic SPARC expression was also associated with worse patient outcome (positive *vs* negative DFS 7.4 *vs* 12.1 mo, *P* = 0.041; OS 14.1 *vs* 25.6 mo, *P* = 0.011) in patients with pancreatic cancer who received gemcitabine as adjuvant CT
Blackford et al[76]	114	mOS,14.2 mo in patients without SMAD4 gene inactivation *vs* 11.5 mo for those with inactivation (*P* = 0.006)
Oshima et al[77]	106	Loss of SMAD4 expression was significantly associated with shorter OS and it was found to be an independent prognostic factor for both OS and DFS
Novel biomarkers		
Xue et al[86]	106	mOS for patients with a higher NEDD9 expression was significantly shorter than that of patients with lower NEDD9 expression. NEDD9 was an independent factor of poor prognosis
Xia et al[88]	80	A higher FoxM1 expression had a significantly shorter survival time compared to patients with lower FoxM1 expression and FoxM1 was found to be an independent factor for survival

mOS: Median overall survival; DFS: Disease-free survival; hENT1: Human equilibrative nucleoside transporter 1; SPARC: Secreted protein and rich in cysteine; CT: Chemotherapy; FOXM1: Forkhead box M1.

out by Perini *et al*[90], prognostic significance of epidermal growth factor receptor (EGFR) overexpression in pancreas cancer was investigated. Univariate analysis showed that positive EGFR expression in tumor tissue had worse survival, as were male gender, portal vein resection, perineural, lymphovascular and peri-pancreatic invasion, positive margins, however, prognostic significance of positive EGFR expression as an independent prognostic factor could not be confirmed in the multivariate analysis. Selected studies associated with molecular and novel biomarkers are listed in Table 4.

CONCLUSION

The overall prognosis associated with pancreatic cancer has not improved over the last 20 years, even if new diagnostic and therapeutic strategies have emerged. So, investigations on predictive factors in pancreatic cancer are needed because these factors should have predictive value in relation to longer survival after surgery than after palliative treatment. In addition to some well-known prognostic factors such as tumor stage, surgical margin, perineural invasion, PS, treatment effect, and CA19-9, recently new prognostic indicators that have an impact on survival of patients with pancreatic cancer have appeared. The prognostic value of operative factors including OBL and PBT, NLR, and molecular markers such as SPARC, hENT1, SMAD4, CTCs, HIF1α, NEDD9 and FOXM1 has recently been shown. Prognostic factors are important and a correct definition of poor prognostic factors may help to guide more aggressive adjuvant or aggressive treatment protocols in patients with pancreatic cancer.

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2014 ADVANCES IN PANCREATIC CANCER

Targeting tight junctions during epithelial to mesenchymal transition in human pancreatic cancer

Daisuke Kyuno, Hiroshi Yamaguchi, Tatsuya Ito, Tsuyoshi Kono, Yasutoshi Kimura, Masafumi Imamura, Takumi Konno, Koichi Hirata, Norimasa Sawada, Takashi Kojima

Daisuke Kyuno, Hiroshi Yamaguchi, Tatsuya Ito, Tsuyoshi Kono, Yasutoshi Kimura, Masafumi Imamura, Koichi Hirata, Department of Surgery, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

Tsuyoshi Kono, Takumi Konno, Takashi Kojima, Department of Cell Science, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

Norimasa Sawada, Department of Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

Author contributions: Kyuno D and Kojima T contributed equally to this work; Yamaguchi H, Ito T, Kono T, Kimura Y, Imamura M, Konno T, Hirata K and Sawada N designed the research; Kyuno D and Kojima T wrote the paper.

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Correspondence to: Takashi Kojima, PhD, Department of Cell Science, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, South 1, West 17, Sapporo 060-8556, Japan. ktakashi@sapmed.ac.jp

Telephone: +81-11-6112111 Fax: +81-11-6112299

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Abstract

Pancreatic cancer continues to be a leading cause of cancer-related death worldwide and there is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of patients with this disease. In pancreatic cancer, some tight junction proteins, including claudins, are abnormally regulated and therefore are promising molecular targets for diagnosis, prognosis and therapy. Claudin-4 and -18 are overexpressed in human pancreatic cancer and its precursor lesions. Claudin-4 is a high affinity receptor of Clostridium perfringens enterotoxin (CPE). The cytotoxic effects of CPE and monoclonal antibodies against claudin-4 are useful as novel therapeutic tools for pancreatic cancer. Claudin-18 could be a putative marker and therapeutic target with prognostic implications for patients with pancreatic cancer. Claudin-1, -7, tricellulin and marvelD3 are involved in epithelial to mesenchymal transition (EMT) of pancreatic cancer cells and thus might be useful as biomarkers during disease. Protein kinase C is closely related to EMT of pancreatic cancer and regulates tight junctions of normal human pancreatic duct epithelial cells and the cancer cells. This review focuses on the regulation of tight junctions *via* protein kinase C during EMT in human pancreatic cancer for the purpose of developing new diagnostic and therapeutic modalities for pancreatic cancer.

Key words: Tight junctions; Claudins; Tricellulin; MarvelD3; Normal human pancreatic duct epithelial cells; Pancreatic cancer; Protein kinase C; Epithelial to mesenchymal transition

Core tip: There is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of pancreatic cancer patients. In pancreatic cancer, some tight junction proteins, including claudins, are abnormally regulated and thus are promising molecular targets for Clostridium perfringens enterotoxin and monoclonal antibodies. Protein kinase C is closely related to epithelial to mesenchymal transition (EMT) of this cancer and regulates tight junctions of normal human pancreatic duct epithelial (HPDE) cells and pancreatic cancer cells. This review focuses on the regulation of tight junctions *via* protein kinase C during EMT in human pancreatic cancer compared to normal HPDE cells.

Original sources: Kyuno D, Yamaguchi H, Ito T, Kono T, Kimura Y, Imamura M, Konno T, Hirata K, Sawada N, Kojima T. Targeting tight junctions during epithelial to mesenchymal transition in human

INTRODUCTION

Pancreatic cancer continues to be a leading cause of cancer-related death worldwide due to late detection, lack of therapeutic targets and ineffective therapies. At the time of diagnosis, few patients with pancreatic cancer present with localized disease amenable to surgical resection, while the remaining patients present with locally advanced or distant metastasis. It exhibits the poorest prognosis of all solid tumors with a 5-year survival rate < 5% and a median survival of 3-6 mo after diagnosis[1]. Thus, there is an urgent need to develop novel diagnostic and therapeutic strategies to reduce the mortality of these patients.

Transition of a cancer cell from an epithelial to mesenchymal morphology leads to increased migratory and invasive properties, and thus facilitates the initiation of metastasis in pancreatic cancer[2,3]. The epithelial to mesenchymal transition (EMT) is characterized by a loss of cell-cell contact and apicobasal polarity. The hallmarks of EMT *in vitro* and *in vivo* include the upregulation of mesenchymal markers, the downregulation of epithelial cell adhesion molecules including tight junction proteins, and dysfunction of the tight junction fence[4,5]. EMT is accompanied by loss of occludin and claudins as well as E-cadherin *via* the Snail family[6-9]. The transcription factor Snail, which has high to moderate expression in 78% of pancreatic ductal adenocarcinoma specimens, appears to promote metastasis and chemoresistance in pancreatic cancer[10,11]. The activation of protein kinase C (PKC) is known to be involved in EMT in various type of cancer including pancreatic cancer. The PKC activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induces EMT in human prostate cancer cells[12] and pancreatic cancer cell line HPAC[13]. Expression of PKCα and PKCδ closely contributes to EMT in colon cancer cells[14,15]. Transforming growth factor-β1 (TGF-β1), which promotes EMT in pancreatic cancer cells[16], induces PKCα in poorly differentiated pancreatic cancer cell line BXPC-3[17].

In several human cancers, including pancreatic cancer, some tight junction proteins are abnormally regulated and therefore promising molecular targets for diagnosis and therapy[18,19]. The current review will focus on the roles of tight junction proteins, including claudins, and PKC signaling with regard to the potential applicability for diagnosis, prognosis and the therapy during EMT in pancreatic cancer.

TIGHT JUNCTION AND ITS PROTEINS

Epithelial cells including pancreatic epithelial cells are bordered by two functionally and biochemically different membranes[20]. This integrity is maintained by intercellular junctional complexes, such as tight junctions, adherent junctions, and desmosomes[21]. Tight junctions are the most apical components of intercellular junctional complexes in epithelial and endothelial cells. They separate the apical and basolateral cell surface domains, maintaining cell polarity (termed the "fence" function), and selectively control solute and water flow through the paracellular space (termed the "barrier" function)[22-25]. They also participate in signal transduction mechanisms that regulate epithelial cell proliferation, gene expression, differentiation and morphogenesis[26]. The tight junction is formed by integral membrane proteins and peripheral membrane proteins. The integral membrane proteins are claudins[27,28], occludin[29], tricellulin[30], marvelD3[31] and junctional adhesion molecules[32] (Figure 1). Peripheral membrane proteins include the scaffold PDZ-expression proteins zonula occludens (ZO)-1, ZO-2, ZO-3, multi-PDZ domain protein-1, membrane-associated guanylate kinase with inverted orientation-1 (MAGI)-1, MAGI-2, MAGI-3, cell polarity molecules atypical PKC isotype-specific interacting protein/PAR-3, PAR-6, PALS-1, and PALS-1-associated tight junction, as well as the non-PDZ-expressing proteins cingulin, symplekin, ZONAB, GEF-H1, aPKC, PP2A, Rab3b, Rab13, PTEN, and 7H6[21,33,34]. These tight junction proteins are regulated by various cytokines and growth factors *via* distinct signal transduction pathways including PKC[35,36].

The claudin family, which consists of at least 27 members, is solely responsible for forming tight junction strands and has four transmembrane domains and two extracellular loops[21,37] (Figure 2). The first extracellular loop is the coreceptor of hepatitis C virus[38] and influences the paracellular charge selectivity[39], and the second extracellular loop is the receptor of Clostridium perfringens enterotoxin (CPE)[40].

Both occludin and tricellulin (marvelD2) contain the tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is present in proteins involved in membrane apposition and concentrated in cholesterol-rich microdomains[41]. The novel tight junction protein marvelD3 contains a conserved MARVEL domain like occludin and tricellulin[31,42].

In general, cancer cells lose their specific functions and polarity with a decrease in the development of tight junctions. It is thought that the loss of tight junction functions in part leads to invasion and metastasis of cancer cells[43].

Tight junction proteins are dysregulated during carcinogenesis and EMT. Expression of some claudin family members is significantly altered by epigenetic regulation in human cancer[44-46].

EXPRESSION PATTERNS AND THE ROLE OF TIGHT JUNCTION PROTEINS IN NORMAL PANCREAS

Several tight junction proteins are expressed in a tissue-specific and organ-specific manner[47-49]. Normal ductal
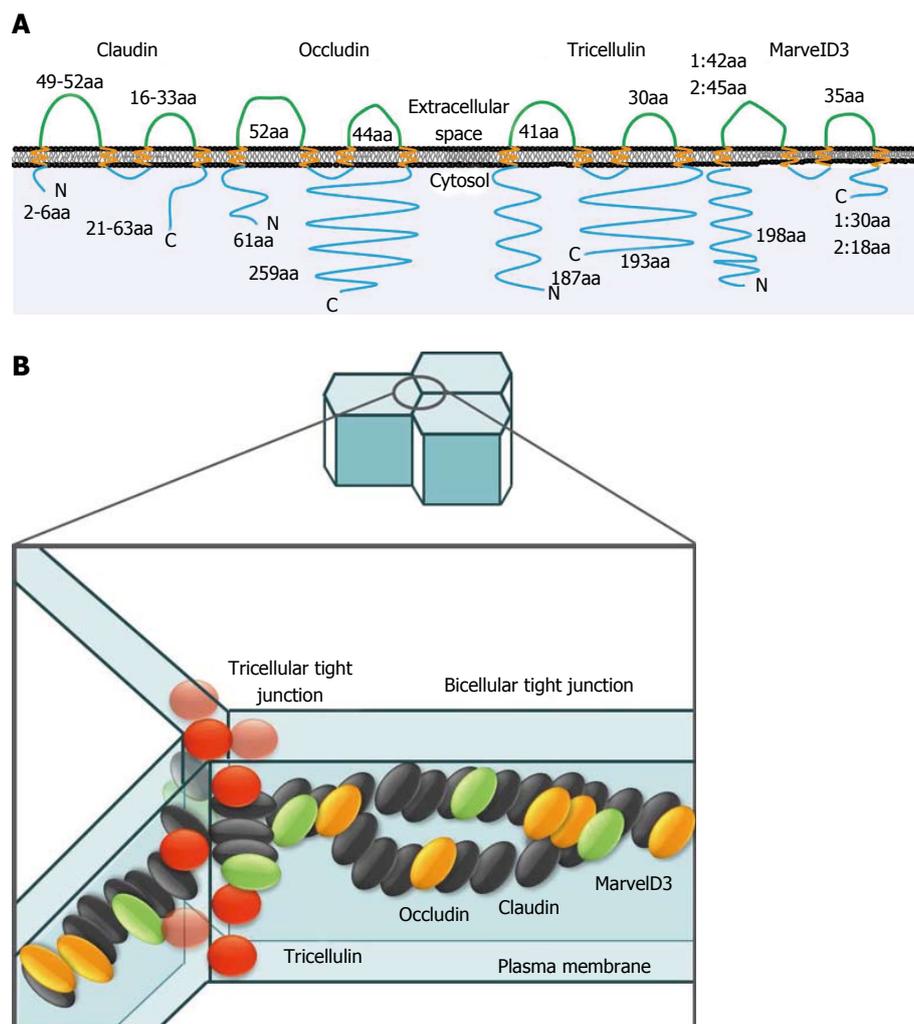
Figure 1 Claudins, occludin, tricellulin, marvelD3 and junctional adhesion molecules. A: Schematic representation of human claudin, occludin, tricellulin, and marvelD3. These molecules contain four transmembrane domains with two extracellular loops. Claudins consist of at least 27 members. Occludin has several variants. MarvelD3 has two isoforms. aa: amino acid; B: Models of tight junction protein locations in paracellular space. The bicellular tight junction is the interface between two cells, whereas the vertex where three cells meet is termed the tricellular tight junction. The tight junction strands within both bicellular and tricellular regions are composed of claudins (black ellipses). MarvelD3 (green ellipses), occludin (orange ellipses), and tricellulin (red spheres) incorporated into claudin-based tight junction strands. Occludin and tricellulin are primarily found at bicellular and tricellular regions, respectively, whereas marvelD3 is present at both sites. Tricellulin is unique in that it is present at the tight junction and along the lateral membrane.

and acinar structures of the pancreas express claudin-1, -2, -3, -4, and -7, whereas endocrine cells within the islets of Langerhans express claudin-3 and -7 (Figure 3)[50,51]. Pancreatic duct cells deliver the enzymes produced by acinar cells into duodenum and secrete a HCO_3^--rich fluid to neutralize gastric acid from the stomach[52]. Tight junctions of the pancreatic duct form the pancreatic ductal barrier. Freeze-fracture analysis of the pancreatic duct reveals that tight junctions contained a parallel array of three to five continuous sealing strands and the pancreatic enzymes cannot leak out from the lumen into the intercellular spaces (Figure 3)[53,54]. Tight junctions of the pancreatic duct are also regulators of physiologic secretion of the pancreas. Pancreatic ductal tight junctions, which is leaky and has the function of selective permeability, may play a role of channels of Na^+ and HCO_3^- *via* paracellular pathway[55,56].

The tight junctions of pancreatic duct epithelial cells and exocrine cells are dynamic structures that can be disrupted by various external stimuli including ductal hypertension[57,58]. The disruption of pancreatic duct tight junctions is an early event in different types of pancreatitis[59-64]. Although dysfunction of tight junctions in pancreatic duct is observed by various pathological conditions, the regulatory mechanisms of tight junctions remain unknown even in normal human pancreatic duct epithelial (HPDE) cells.

EXPRESSION PATTERNS OF TIGHT JUNCTION PROTEINS IN PANCREATIC CANCER

The tight junction protein expression pattern varies be-
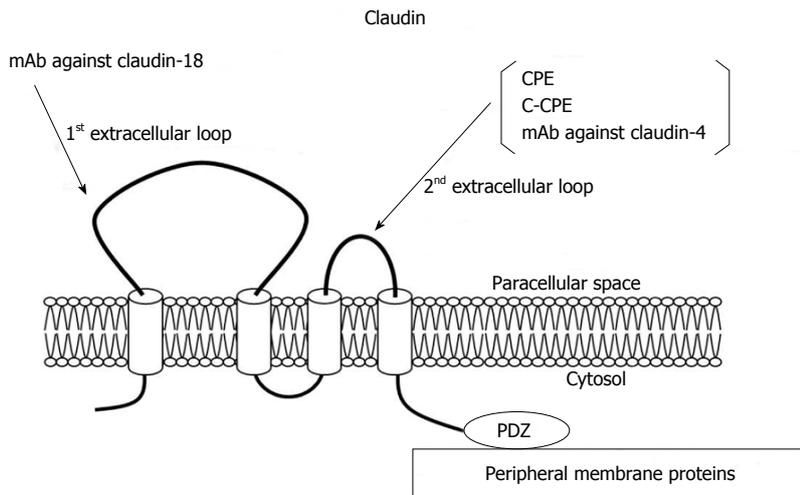
Figure 2 Structures of claudins. The first extracellular loop of claudin-18 targeted for therapy using monoclonal antibodies and the second extracellular loop of claudin-4 targeted for therapy using monoclonal antibodies, Clostridium perfringens enterotoxin and C-Clostridium perfringens enterotoxin.
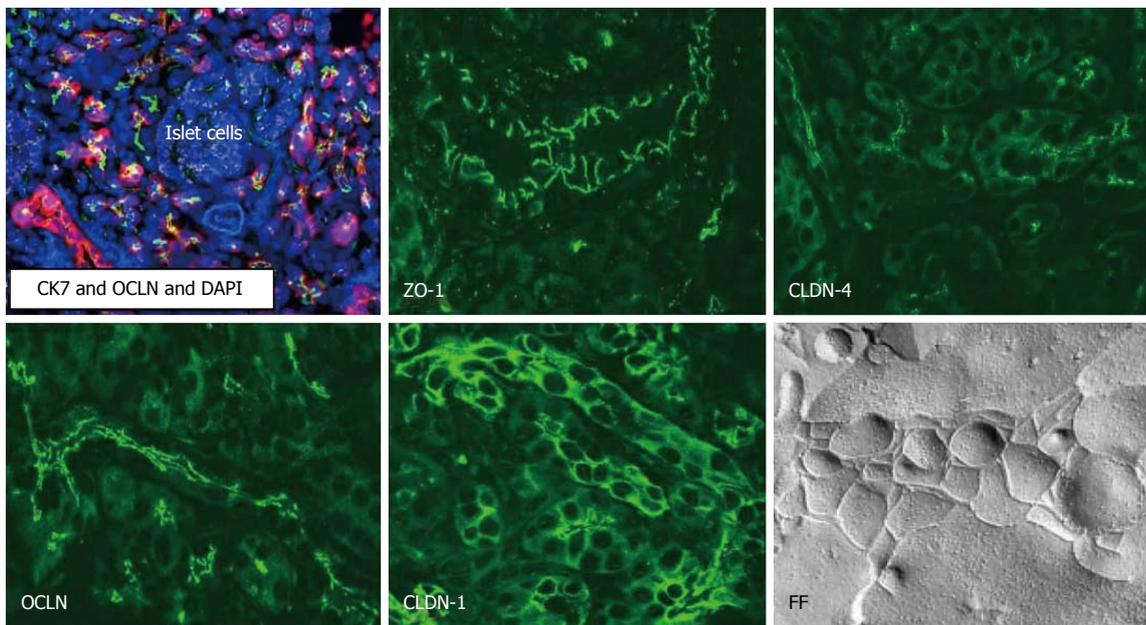
Figure 3 Localization and structures of tight junctions in normal human pancreas. In normal pancreatic ducts which express CK (Cytokeratin)7, occludin (OCLN), ZO-1 and claudin (CLDN)-1, -4 are observed by immunostaining. In freeze-fracture (FF) replica, well-developed tight junction strands are observed in normal pancreas.

tween normal pancreatic tissue and pancreatic cancer. Claudin-1, -4, -7 and -18 are positive in pancreatic adenocarcinoma, whereas endocrine tumors are negative for claudin-1 and -4. Claudin-3 and -7 proteins are detected in endocrine tumors, whereas claudin-13 is negative in ductal adenocarcinoma[18,50,51]. Claudin-1, -2 and -4 are detected in exocrine tumors[65]. In borderline cystic tumors the level of claudin-1, -4 and -7 protein expression is between that of benign and malignant tumors[65]. This supports the sequential development theory regarding mucinous cystic tumors.

Liver metastasis of pancreatic cancer is strongly positive for claudin-4, weakly positive for claudin-1, and negative or faintly positive for claudin-7[66]. It is interesting

that claudin-3 is positive in liver metastasis of pancreatic cancer whereas claudin-3 staining is not detected in primary pancreatic cancer[50,66].

A study investigating ZO-1 in pancreatic cancer showed that expression of ZO-1 was increased in pancreatic adenocarcinoma samples in comparison with normal samples[67]. In pancreatic cancer cells, ZO-1 protein translocalizes from apical and apicolateral areas to the cytoplasm and nucleus, and translocation of ZO-1 is involved in the induction of invasion through epidermal growth factor receptor (EGFR) activation[68].

We established human telomerase reverse transcriptase-transfected HPDE cells as models of normal pancreatic duct epithelial cells[51]. The hTERT-HPDE cells

are positive for HPDE cell markers such as CK7, CK19 and carbonic anhydrase isozyme 2 and express epithelial tight junction molecules claudin-1, -4, -7 and -18, occludin, tricellulin, marvelD3, JAM-A, ZO-1, and ZO-2[51]. The expression patterns of tight junction molecules in the hTERT-HPDE cells are similar to those of pancreatic tissues *in vivo*[51].

CLAUDIN-1 IN NORMAL PANCREATIC DUCT AND CANCER

Claudin-1 is expressed in various types of epithelial cells, and plays an important role in epithelial cell polarity and cancer invasion and metastasis[69-72]. However, its role remains controversial far in various cancers. In pancreatic cancer, claudin-1 expression is responsible for tumor necrosis factor α-dependent cell growth signals that lead to apoptosis and the inhibition of cell proliferation[73]. Claudin-1 is localized at the cell membranes of normal pancreatic ducts and well-differentiated pancreatic carcinoma, whereas in poorly differentiated pancreatic carcinoma it is weakly detected in cytoplasm[74].

EMT is associated with the simultaneous repression of the genes encoding E-cadherin, claudins and occludin[8]. The transcription factors Snail and Slug, which play a central role in EMT, bind to the E-box motifs present in the claudin-1 promoter and have a critical negative regulatory role in malignant cancer cell lines that express low levels of the claudin-1 transcript[8,75]. Treatment with TGF-β1 induces EMT in pancreatic cancer cells and TGF-β upregulates Snail and downregulates claudin-1, -4 and occludin in PANC-1 cells[74]. Taken together, this indicates that claudin-1 may be a potential biomarker for the development of pancreatic cancer. Thus further investigation of the significance of claudin-1 in pancreatic cancer cells and normal pancreatic duct epithelial cells is required.

CLAUDIN-4 IN NORMAL PANCREATIC DUCT AND CANCER

DNA microarray, immunohistochemical, and quantitative real-time reverse transcription-polymerase chain reaction analyses have provided evidence that claudin-4 is upregulated in pancreatic cancer tissues[76]. Furthermore, claudin-4 is also overexpressed in pancreatic intraepithelial neoplasia (PanIN), intraductal papillary neoplasia (IPMN), and mucinous cystic neoplasia (MCN), and is correlated with the histological tumor grade in both IPMN and MCN[77,78]. On the other hand, overexpression of claudin-4 decreases the invasiveness and metastatic potential of pancreatic cancer cells *in vitro*[19]. Patients with high expression of claudin-4 mRNA and protein survive longer than those with low claudin-4 expression[79].

Claudin-4 is also a high-affinity receptor of CPE[80]. The 35-kDa polypeptide CPE causes food poisoning in humans, binds to its claudin receptor, and then causes changes in membrane permeability *via* formation of a complex on the plasma membrane followed by the induction of apoptosis[81]. Full-length CPE with a direct cytotoxic effect and the COOH-terminal receptor-binding domain of CPE (C-CPE) without a cytotoxic effect are employed as selective treatment and drug delivery systems against claudin-4 expressing pancreatic tumors[82,83].

CPE induces an acute dose-dependent cytotoxic effect in claudin-4-expressing nude mouse xenografts of PANC-1, which is a poorly differentiated pancreatic cancer cell line[82,84]. In the pancreatic cell lines PANC-1, BXPC-3, HPAF-Ⅱ and HPAC, claudin-4 is found not only at the apicalmost regions but also at basolateral membranes[85]. When these pancreatic cancer cell lines are treated with CPE, it induces dose-dependent cytotoxic effects in all of them[85]. Furthermore, in HPAC cells, the cytotoxicity of CPE is significantly decreased by knockdown of claudin-4 by siRNAs[85].

In hTERT-HPDE cells cultured with 10% FBS, claudin-4 is localized at the apicalmost regions, which are tight junction areas[85]. When hTERT-HPDE cells cultured with 10% FBS in which the expression of claudin-4 protein is as high as in pancreatic cell lines in Western blotting, are treated with CPE, cytotoxicity is not observed even at high concentrations of CPE[85]. These findings suggest that, in pancreatic cancer cells, CPE binds to the free second extracellular loop of claudin-4 outside of tight junctions and that, in normal HPDE cells, it cannot bind to that of claudin-4 in tight junction areas.

EFFECT OF C-CPE TARGETING CLAUDIN-4 AGAINST PANCREATIC CANCER

The functional domains of CPE can be separated into a receptor-binding region (C-terminal of CPE, C-CPE) and cytotoxic region (N-terminal of CPE). C-CPE is a C-terminal fragment composed of the CPE amino acids 184 to 319[80]. The receptor binding region of CPE has been reported to be in the C-terminal 30 residues (amino acids 290 to 319) of CPE[86].

C-CPE is a nontoxic molecule that disrupts the tight junction barrier function and enhances cellular absorption[87]. It enhances the effectiveness of clinically relevant anticancer agents such as Taxol and carboplatin against cancer cells[88]. In our study, when HPAC cells were treated with C-CPE, the barrier function was markedly decreased at a nontoxic concentration of C-CPE and recovered in the absence of C-CPE (personal data). C-CPE may enhance the effectiveness of clinically relevant chemotherapies in pancreatic cancer.

The development of molecular imaging approaches using tissue- and cell-specific tracers plays a crucial role to improve early diagnosis and therapy in cancer. Claudin-4 is utilized as a target for imaging of pancreatic cancer. Non-cadmium-based quantum dots bioconjugated to claudin-4 monoclonal antibodies are used as highly ef-

ficient, nontoxic optical probes for imaging live pancreatic cancer cells *in vivo* and *in vitro*[89]. C-CPE labelled with a cyanine dye with novel optical imaging methods, 2D planar fluorescence reflectance imaging technology and 3D fluorescence-mediated tomography, enables noninvasive visualization of claudin-4 positive pancreatic cancer and its precursor lesions[90]. Furthermore, it is thought that C-CPE can be used as a carrier for other bacterial toxins to claudin-4-positive cancer cells. A claudin-4-targeting antitumor molecule that consisted of C-CPE fused to protein synthesis inhibitory factor derived from *Pseudomonas aeruginosa* exotoxin or diphtheria toxin fragment A (DTA) were especially toxic to claudin-4 positive cancer cells in vivo and *in vitro*[83,91,92].

CLAUDIN-7 IN NORMAL PANCREATIC DUCT AND CANCER

Claudin-7 is expressed in various types of epithelial cells and directly interacts with EpCAM, forming a complex with CD44 variant isoforms and tetraspanins outside of tight junction areas[93,94]. Furthermore, EpCAM is one of the surface markers in pancreatic cancer stem cells[95], and claudin-7 regulates the EpCAM-mediated functions in tumor progression such as proliferation, migration, and anti-apoptosis[96,97]. Claudin-7 supports tumorigenic features of EpCAM by provoking EpCAM cleavage and its cotranscription factor activity, and is directly engaged in motility and resistance to apoptosis in rat pancreatic cancer[98].

In human pancreatic ductal adenocarcinoma, there is a gradual decline in membrane-bound expression of claudin-7 immunoreactivity in parallel with the degree of tumor differentiation[99]. Claudin-7 expression also appears to be inversely associated with the gland size in tumors, with large neoplastic glands displaying more frequent claudin-7 positivity than smaller glands[99]. There is no association between claudin-7 and tumor size, the presence of nodal metastases or survival of the patients, indicating that while expression of claudin-7 is related to differentiation of ductal pancreatic adenocarcinoma it does not influence tumor progression[99].

In a human pancreatic cancer cell line and hTERT-HPDE cells, ELF3 is associated with claudin-7[51]. ELF3 belongs to the ELF (E74-like factor) subfamily of the ETS transcription factors, but it is distinguished from most ETS family members by its expression pattern, which is specific in epithelial tissues of the lung, liver, kidney, pancreas, prostate, small intestine, and colon mucosa[100]. ELF3 controls intestinal epithelial differentiation[101]. It is reported that the expression of claudin-7 in epithelial structures in synovial sarcoma is regulated by ELF3[102]. Thus, the expression of claudin-7 and its regulation *via* ELF3 may be important as potential therapeutic targets for pancreatic cancer.

CLAUDIN-18 IN NORMAL PANCREATIC DUCT AND CANCER

In pancreatic cancer, claudin-18 is as highly expressed as claudin-4[18]. Claudin-18 has two alternatively spliced variants, claudin-18a1 and claudin-18a2, which are highly expressed in the lung and stomach, respectively[103]. Claudin-18a2 is activated in a wide range of human malignant tumors, including gastric, esophageal, pancreatic, lung, and ovarian cancers, and can be specifically targeted by monoclonal antibodies against the first extracellular loop[44]. Claudin-18 is highly expressed in PanIN, IPMN, MCN, pancreatic duct carcinoma, and metastases of pancreatic cancer, and serves as a diagnostic marker[18,78,99,104-106]. Neuroendocrine neoplasia is found positive with low rates[105]. Thus, claudin-18 could be useful as a putative marker and therapeutic target for neoplasia of the pancreas. Furthermore, because claudin-18 expression is most pronounced in well-differentiated pancreatic cancers, and patients with high expression of claudin-18 survive longer than those with low claudin-18 expression[18], its expression level may also have prognostic implications for patients with pancreatic cancer.

TRICELLULIN IN NORMAL PANCREATIC DUCT AND CANCER

Tricellulin was identified as the first marker of the tricellular tight junction, which formed at the meeting points of three cells[30]. It is required for the maintenance of the transepithelial barrier and expressed in both the normal pancreatic duct and pancreatic cancer[30,107,108]. It is one of three members of the tight junction-associated MARVEL protein family. The other two members are occludin and marvelD3[31,42]. Occludin and tricellulin are present at bicellular and tricellular tight junctions, respectively, whereas marvelD3 is present at both sites[31,42]. Both normal and neoplastic pancreatic exocrine tissues express tricellulin, whereas no expression is seen in normal or neoplastic endocrine cells[108]. Tricellulin expression in pancreatic ductal adenocarcinomas shows a significant negative correlation with the degree of differentiation[108].

Tricellulin expression in tricellular tight junctions is strongly regulated together with the barrier function *via* the c-Jun N-terminal kinase (JNK) transduction pathway[109]. Activation of JNK promotes the development of various tumors[110-112]. Furthermore, JNK inhibitors decrease the growth of human and murine pancreatic cancers *in vitro* and *in vivo*[113]. Tricellulin expression and the barrier function are upregulated together with the activity of phospho-JNK by treatment with the JNK activator anisomycin in HPAC cells[109]. In hTERT-HPDE cells, tricellulin expression is significantly increased by all JNK activators, similar to the response in HPAC cells[109].

JNK may be involved in the regulation of tight junctions, including tricellulin expression and the barrier function in normal pancreatic duct epithelial cells, and may be a potential therapeutic target for pancreatic cancer.

MARVELD3 IN NORMAL PANCREATIC DUCT AND CANCER

MarvelD3, the novel tight junction protein, is transcriptionally downregulated in poorly differentiated pancreatic cancer cells, whereas it is maintained in well-differentiated human pancreatic cancer cells and normal pancreatic duct epithelial cells[114]. Furthermore, marvelD3 is transcriptionally downregulated in Snail-induced EMT during the progression of pancreatic cancer[114]. Therefore, marvelD3 could be a new marker during pancreatic cancer progression. However, little is known about the detailed role of marvelD3 in epithelial tight junctions and how it is regulated in various types of cells, including normal pancreatic duct epithelial cells and pancreatic cancer cells.

ROLE OF PKC IN TIGHT JUNCTIONS DURING EMT IN NORMAL PANCREATIC DUCT AND CANCER

PKC belongs to the family of serine-threonine kinases and regulates various cellular functions[115]. It has been shown to induce both assembly and disassembly of tight junctions depending on the cell type and conditions of activation[116-118]. At least 12 different isozymes of PKC are known and can be subdivided into three classes (classic or conventional, novel and atypical isozymes) according to their responsiveness to activators[119,120]. The levels of PKCα, PKCβ1, PKCδ and PKCι are higher in pancreatic cancer, whereas that of PKCε is higher in normal tissue[121,122]. In pancreatic cancer, tumorigenicity is directly related to PKCα expression, as demonstrated by decreased survival when it is overexpressed[123]. The increased level of PKCα is also associated with pancreatic cancer cell proliferation[124].

Tight junction proteins are regulated by various cytokines and growth factors *via* distinct signal transduction pathways including PKC[35,36]. In various cancer cells, the regulation of tight junctions *via* PKC pathway is reported. The assembly of ZO-1 and occludin is involved in PKC-dependent signaling in gastric cancer cells[125]. The activation of c-Abl-PKCδ signaling pathway is critically required for the claudin-1-induced acquisition of the malignant phenotype in human liver cells[72]. PKC activation causes an increase in claudin-1 transcription and claudin-1 appears to contribute to cell invasion in human melanoma cells[126]. PKCε activation regulates an α5 integrin-ZO-1 complex and correlates with invasion and unfavorable prognosis in lung cancer cells[127].

We have previously reported that the regulation of tight junctions in normal human pancreatic duct epithelial cells and pancreatic cancer cells is closely associated with PKC and PKC-induced transcriptional factors[13,51,74,104,109,128]. To confirm whether the PKC signal pathway was closely associated with the regulation of tight junctions, hTERT-HPDE cells and pancreatic cancer cells were treated with the PKC activator TPA and the specific PKC isoform inhibitors. Treatment with TPA enhanced expression of claudin-1, -4, -7, and -18, occludin, JAM-A and ZO-1, -2[51]. The upregulation of claudin-4 by TPA was prevented by a PKCα inhibitor and the upregulation of claudin-7, occludin, ZO-1 and ZO-2 was prevented by a PKCδ inhibitor[51]. In HPAC cells, tricellulin was in part regulated *via* PKCδ and PKCε pathways[109], and the expression of claudin-18 and localization of claudin-4 and occludin were in part regulated *via* a PKCα pathway[13,104,128]. Claudin-18 mRNA and protein, indicated to be claudin-18a2, were markedly induced by TPA in well- and moderately differentiated human pancreatic cancer cell lines HPAF-Ⅱ and HPAC and hTERT-HPDE cells[104]. The upregulation of claudin-18 by TPA in human pancreatic cancer cell lines was prevented by inhibitors of PKCδ, PKCα and PKCε, whereas the upregulation of claudin-18 by TPA in hTERT-HPDE cells was prevented by inhibitors of PKCδ, PKCα and PKCθ[104].

On the other hand, a PKCα inhibitor enhances sensitivity of HPAC cells to CPE by preventing mislocalization of claudin-4[13], and prevents downregulation of claudin-1 during EMT of pancreatic cancer cells[74]. The TGF-β-PKCα-PTEN cascade is a key pathway for pancreatic cancer cells to proliferate and metastasize[129]. The PKC may be a useful target for pancreatic cancer therapy[119] and PKCα inhibitors may be potential therapeutic agents against the malignancy of human pancreatic cancer cells[130]. Further study of the tight junctions of normal HPDE cells and pancreatic cancer cells *via* PKC pathways including isoforms is important for not only physiological regulation of tight junction molecules but also for therapeutic targeting of pancreatic cancer cells. In addition to PKC pathway, other signaling pathways including Ras/ERK1/2, Smad/STAT3, Notch, Wnt and Src are closely related to EMT of pancreatic cancer[131-135]. However, the regulation of tight junctions in normal pancreatic duct and pancreatic cancer *via* these signal pathways remain unknown.

CONCLUSION

The signaling pathways including PKC regulate tight junctions during EMT in pancreatic cancer. By using hTERT-HPDE cells, we found that the expression of tight junction proteins in normal HPDE cells was regulated by various factors. For developing new diagnostic and therapeutic modalities *via* tight junction molecules in pancreatic cancer, it is necessary to investigate the profile and the regulation of tight junctions in normal HPDE cells as well as pancreatic cancer cells.

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Novel therapeutic targets for pancreatic cancer

Shing-Chun Tang, Yang-Chao Chen

Shing-Chun Tang, Yang-Chao Chen, School of Biomedical Sciences, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong, China
Yang-Chao Chen, Shenzhen Research Institute, The Chinese University of Hong Kong, Hong Kong, China
Author contributions: Chen YC and Tang SC wrote the paper.
Correspondence to: Yang-Chao Chen, Professor, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China. frankch@cuhk.edu.hk
Telephone: +852-39435728 Fax: +852-26035123
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Abstract

Pancreatic cancer has become the fourth leading cause of cancer death in the last two decades. Only 3%-15% of patients diagnosed with pancreatic cancer had 5 year survival rate. Drug resistance, high metastasis, poor prognosis and tumour relapse contributed to the malignancies and difficulties in treating pancreatic cancer. The current standard chemotherapy for pancreatic cancer is gemcitabine, however its efficacy is far from satisfactory, one of the reasons is due to the complex tumour microenvironment which decreases effective drug delivery to target cancer cell. Studies of the molecular pathology of pancreatic cancer have revealed that activation of KRAS, overexpression of cyclooxygenase-2, inactivation of p16^{INK4A} and loss of p53 activities occurred in pancreatic cancer. Co-administration of gemcitabine and targeting the molecular pathological events happened in pancreatic cancer has brought an enhanced therapeutic effectiveness of gemcitabine. Therefore, studies looking for novel targets in hindering pancreatic tumour growth are emerging rapidly. In order to give a better understanding of the current findings and to seek the direction in future pancreatic cancer research; in this review we will focus on targets suppressing tumour metastatsis and progression, KRAS activated downstream effectors, the relationship of Notch signaling and Nodal/Activin signaling with pancreatic cancer cells, the current findings of non-coding RNAs in inhibiting pancreatic cancer cell proliferation, brief discussion in transcription remodeling by epigenetic modifiers (*e.g.*, HDAC, BMI1, EZH2) and the plausible therapeutic applications of cancer stem cell and hyaluronan in tumour environment.

Core tip: Some of the targets discussed here have been discovered to enhance the effectiveness of gemcitabine upon co-administration of the corresponding agents, for instance, hyaluronidase can deplete hyaluronan in stromal region to enhance gemcitabine delivery. Besides, some signaling molecules, *e.g.*, RalGEF-RAl, Rac1, and PAR2 are being targeted to suppress metastasis. Tumour proliferation is limited upon DR5 activated apoptosis and others promising therapeutic areas like epigenetic modifiers; IAP, miR, lncRNA, and cancer stem cells-tumour microenvironment will also be discussed.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the last two decades because of various obstacles in its treatment[1]. Late and poor prognosis are two

of the causes of high fatality rate[2]. Patients diagnosed having pancreatic cancer are usually at their very late stage and spreading of the highly metastatic pancreatic cancer cell into the lymphatic system and vicinal organs limited the choices of effective treatments[3].

Gemcitabine is the current standard chemotherapy for pancreatic cancer[4], however, due to the complex tumour microenvironment[5] and high metastatic property of pancreatic cancer. The effectiveness of gemcitabine in treating pancreatic cancer is unsatisfactory. Studies of targeting the molecular pathology have been carried out to quest for more potential targets; for instance, activation of oncoprotein V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)[6], overexpression of cyclo-oxygenase-2[1], inactivation of p16[INK4A][6] and loss of p53 activities[6] mark the onset of pancreatic cancer. Because, the treatments of targeting these molecules can enhance the efficacy of gemcitabine in pancreatic cancer[3], these imply that combinatorial therapies may be the future direction in treating pancreatic cancer[7]. Therefore, in the following context of this review we are going to briefly evaluate plausible therapeutic targets, in terms of the molecular and cellular level which covers the roles of several signal transducers, signaling pathways, surface proteins, receptor proteins, non-coding RNA, epigenetic modifiers and tumour microenvironment in driving pancreatic cancer and to explore any possibilities of combinatorial therapy among them.

SIGNAL TRANSDUCERS

CTHRC1
Collagen Triple Helix Repeat Containing-1 (CTHRC1) is a secretory protein[8], which participates in vascular remodulation through limiting collagen matrix deposition[9], and also morphogenesis but most importantly enhancing cell migratory ability and adhesiveness in tumour cells[8]. CTHRC1 is found expressed in a wide spectrum of human cancer cells, and is in particular found highly expressed in pancreatic cells[10]. The CTHRC1 protein is found highly expressed in invasive melanoma but weakly expressed or absent in benign nevi or non-invasive melanoma[8]. Over expression of CTHRC1 in pancreatic cancer has enhanced the tumour cells migration and metastatic properties; studies of using induced hyper CTHRC1 expressed pancreatic cancer cell, MiaPaCa-2-CTHRC1 and shRNA-CTHRC1 suppressed pancreatic cancer cells, BxPC3 and Panc1, are used to evaluate CTHRC1 on pancreatic cancer cells metastatic in *in vivo* mice model[10]. The result has revealed a wider metastatic spread of hyper CTHRC1 expressed pancreatic cancer cell to secondary organs while the hypo CTHRC1 expressed pancreatic tumour cells has reduced tumour cells spreading to neighboring organs when compared with the tumour cells transfected with control shRNA[10]. The phosphorylation of Focal adhesion kinase (FAK)-steroid receptor coactivator (Src) cascade and extracellular signal-regulated kinases (Erk) are the causes of the enhanced

metastasis[10], it is found that the binding of CTHRC1 onto the wingless-type MMTV integration site family protein, member 5A (Wnt5a) can stabilize the Wnt receptor complex[11] and the facilitated binding of the Wnt5a into its Wnt receptor complex will activate paxillin which leads to phosphorylation of Src-FAK signaling cascade and Erk[10], as both Src and Erk signaling pathways could lead to tumour progression and enhanced motility[12], overexpression of CTHRC1 has increased the phosphorylation of Src and Erk, and vice versa[10], these indicating the CTHRC1 plays a critical role in controlling pancreatic tumour cell adhesiveness and metastasis. Besides, activating the fore mentioned kinases, CTHRC1 is reported to repress the production of collagen I into the stromal environment of pancreatic cancer[8], supporting of its role as a cancer metastasis enhancing gene.

As suppressing CTHRC1 can reduce the metastatic and motility of pancreatic cancer cell, future studies can investigate on the feasibility of combining CTHRC1 targeted therapy with current anti pancreatic cancer drugs. CTHRC1 appears as a promising target in sequestering pancreatic cancer from spreading to neighboring organs, however, whether it could sensitize the tumour cells to current anti-cancer treatments in pancreatic cancer is not yet published. CTHRC1 would be a more promising target if it is proved to sequester pancreatic cancer during chemotherapy, providing a higher chance in elimination of tumour cells in the patient.

RAC1
RAS-related C3 botulinum toxin substrate 1 (Rac1)[13] is found to be an important factor in regulating pancreatic islet morphogenesis[14], failure of cell spreading has been reported on gelatin-coated culture by blocking Rac1 in isolated islet cells[14]. Apart from its vital role in directing organogenesis, Rac1 is one of the Rat sarcoma (Ras) effectors[15] and is being overexpressed in pancreatic cancer[16]. It has been found diminishing the formation of acinar-ductal metaplasia (ADM), pancreatic intraepithelial neoplasia (PanIN) and tumours when its expression is ablated in K-Ras[G12D] induced pancreatic ductual adenocarcinoma (PDAC) mice model[15]. In cancer biology, Rac1 is found to promote tumour migration and metastasis through lamellipodia production[17]. Studies of targeting Rac1 may be beneficial in slowing down the spreading of pancreatic cancer cells.

Two guanine nucleotide exchange factors (GEFs) have been reported activating Rac1, dynamin 2 (Dyn2) has been reported regulating Rac1 in an undefined mechanism[15]; Dyn2 is found associated with vav 1 guanine nucleotide exchange factor (Vav1) in coimmunoprecipitation, an onco-protein acts as a guanine nucleotide exchange factor (GEF) in Rac1 activation, and Vav1 is stabilized by the degradation of lyzozyme and heat shock cognate 70 upon binding with Dyn2[15]. Truncated form of Dyn2 has found unable to associate with Vav1 and leading to reduced activation of Rac1 by 50%[15]. However, cell lines deficit in Vav1 expression (*e.g.*, Panc1) would

undermine this therapeutic direction[15]. Another GEF, T lymphoma invasion and metastasis 1 (Tiam1), which is reported as an oncogene and associated with various cancers, Tiam1 directs Rac1 to enhance tumour proliferation and metastasis through the Wnt signaling pathway, however, suppressions of Tiam1 and Rac1 will lead to the activation of another oncoprotein, RhoA, which also promotes pancreatic tumour cells aggressiveness and metastasis[17]. The tumour growth and long term survival are significantly suppressed and enhanced respectively, upon simultaneous inhibition of Rac1 and RhoA[17]. These revealed the invasiveness and tumour migration of pancreatic tumour cells are under complex controls, balanced Rac1 and RhoA expression level is suggested to be one of those[17], and the possibility of the participation of Dyn2 in between Rac1 and RhoA, as the effect of reduction of activated Rac1 in truncated Dyn2 experiment on RhoA is unknown.

However, when shifting to microRNA research, microRNA-124 (miR-124) is found able to suppress Rac1 mRNA and protein levels in pancreatic cancer cells, through the binding onto 3'-UTR of the Rac1 mRNA[16]. Although the suppression of RhoA by miR-124 is yet to be determined; miR-143 is found able to suppress Rac1 and RhoA at the same time, producing a decreased tumour migration result in a pancreatic tumour cell xenograft model[18].

From the recent findings, Rac1 is difficult to target and obtain therapeutic value, owning to switching on another oncoprotein RhoA, however, the discovery of miR-143 is exemplifying; microRNA could be the way out in tackling target that is similar to Rac1 which has an antagonist carries the similar tumour proliferative and metastasis function. It is worth to investigate on how miR-143 suppressing this "double fused" system in pancreatic tumour metastasis enhancement and its effect on long term survival.

RalGEF-Ral effector signaling network

Ras-like guanine nucleotide exchange factors (RalGEFs) and Ras-like (Ral) protein (which is also named as Ral small GTPase) have drawn increasing attention in cancers mediated by Ras, because RalGEFs are one of the direct effectors of activated Ras[19] and the discoveries of the important roles of Ral proteins in tumourigenesis and metastasis[20], however, the exact mechanism of the signaling network requires further studies to complete. There are more than four kinds of RalGEFs (*e.g.*, RalGDS, Rgl1, Rgl2, and Rgl3) and two homologues of Ral are found, Ral-A and Ral-B, in which they share same nucleotide sequence but differ in 82% of amino acid sequence[19-21]. It is known that activated Ras will activate RalGEFs and in turn the activated RalGEF will convert the GDP bound Ral into GTP bound Ral, the activated Ral GTPase will then activate its downstream targets, for instance RalBP1, filamin, PLCδ1, PLD1, *etc.*, bringing out the corresponding biological responses[19]. Although the general mechanism is elucidated nowadays, the exact

RalGEFs activating RalA and RalB are remain unknown, so do the identity of the exact Ras proteins in activating a particular RalGEFs[19].

There are two homologues of Ral small GTPase which are named RalA and RalB, their roles are distinct in tumourigenesis[19], but are seemingly overlapped in metastasis and invasiveness[21], ubiquitinated form of RalA and RalB have been found and it is in a non-degradative manner for selective localization modulation and functional regulations of Ral[22]. Studies have shown that mutated RalA in a constitutively active state can cause transformation of human cells but not in the same mutant of RalB[23], stable suppression of RalA in pancreatic cancer cells has brought a significant inhibition in the anchorage-independent growth[19], and inhibition of RalA can delay the tumourigenesis K-Ras mutants PDAC in mouse model[19], and the binding of RalA onto RalBP1 or Sec5 is found crucial in Ras - mediated transformation[23]. Aurora A kinase (AAK) is a kind of RalA inhibitors which prevents RalA phosphorylation, in fact an AAK, MLN8237 has been entered phase Ⅲ clinical trials, and such targeting is not effective in suppressing RalA signaling[19].

On the other hand, suppression of RalB alone does not reduce tumourigenesis and transformation but bringing a more pronounced effect in metastatic tumour growth suppression when compared to RalA inhibited alone pancreatic cell lines. In addition, enhanced apoptosis in RalB suppressed cells in suspension state[24]; these are suggesting RalB has a more significant role in the control of metastatic growth of cancer cells than RalA[21]. However, when abrogating the expression of either RalA or RalB in pancreatic cell lines, reduced invasiveness is observed in some pancreatic cancer cells with RalA or RalB suppression but not in all kinds of pancreatic cancer cells, *e.g.*, reduced invasiveness is observed in RalA and RalB suppressed Capan-1 cell line, while in Panc-1 cell line suppressed RalA boosted the cancer cell invasiveness and RalB can bring a reduced invasiveness, and in T3M4 cell line RalB suppression cannot bring down the cancer cell invasiveness but RalA suppression can bring a reduced invasiveness[21]. Thus, RalA and RalB may participate in the control of the invasiveness of pancreatic cancer cells, but there should be some other signaling pathways in control to this tumour malignancy, as the invasiveness reduction cannot be observed in all types of pancreatic cancer cell lines[21]. In regard to the observations, RalB has been suggested in maintaining the viability of the cancer cells in the circulatory system and ensuring tumour cells invasiveness to other organs[21].

Nevertheless, the localization of Ral proteins may also have their roles in the control of the cancer malignancies and is in relation to their ubiquitination and phosphorylation status, as de - ubiquitination of RalA in lipid raft microdomains is reported at the loss of cell-matrix interactions, and ubiquitination of RalA promotes lipid raft microdomains exposure on the cell membrane when the cell got re-adhered[22]. Since lipid raft microdomains served as the platform for various signaling path-

ways, when cancer cell is in detached state, its growth is inhibited due to the loss of related signaling cascade in the lipid raft microdomains[24,25]. Therefore, the prevention of the re - exposure of the lipid raft microdomains onto the membrane can be a direction in the RalGEF - Ral signaling cascade for inhibiting the cancer metastasis by targeting the ubiquitination and activation of RalA.

All in all, the Ral proteins in the RalGEF - Ral signaling cascade play important roles in the control of cancer malignancies, targeting the RalGEF would seem to be efficient in shutting down the transduction of the signaling cascade, however, the question of the availability of inhibitors to RalGEF is concerning, as it is a Ras like signaling molecules, the design of an effective inhibitor to RalGEF may not be easy, and the effectors downstream of this signaling cascade should be closely investigated to aid the discovery of inhibitors that can block the signal transduction downstream of this pathway.

SIGNALING PATHWAYS

Notch signaling pathway

Notch signaling is found to be an important pathway in pancreas development, however the exact mechanism of how Notch regulates pancreatic development and the effectors it recruits are not fully understood[26]. Notch signaling pathway has been reported to maintain a pool of pancreatic progenitor cells at the early stage of pancreatic development, and governs pancreatic ductal cell differentiation which found to be triggered by the intensity of the Notch activation[26]. Implying Notch signaling mediates different effectors depends on cell type, and the stage of organogenesis.

In the pancreatic cancer, Notch signaling molecules are over-expressed[26] and could produce oncogenic, anti-tumour, and drug resistance[27] activities basing on the cellular context[26]. In an ADM study using mouse PDAC model has shown that subject carrying mutant KRAS[28], Notch is constitutively activated and up-regulated in the absence of EGFR[28], while in wild type KRAS carrier, Notch activation requires EGFR activation to induce ADM[29]. Implying the mutation of Ras could alter the activation pathway of Notch. Moreover, the anti-tumour activity of Notch signaling is brought out by Notch2 receptor deletion in mutant KRAS carrier[29], which showed PanIN development is inhibited and subject survival is risen[29]. For the same model, deletion of Notch1 resulted an opposite effect, PanIN development is accelerated and subject median survival is decreased[28,30]. As these two Notch receptors are localized in different compartment of a pancreatic cell, and the exact location of them is not yet concluded[26]. Thus, studying the distribution of Notch1 and Notch2 in pancreatic cell may help to understand their roles in pancreatic cancer and the effectors downstream of this signaling pathway.

As the functions of the Notch1 and Notch2 receptors appeared to be distinct and the involvement of EGFR for activation, the roles of Notch receptors may act as

the decision maker in deciding how the cell behave according to the external environment. Due to the complex environment during cancer development, figuring out the roles of Notch at each stage of the pancreatic cancer development will definitely help sorting out targets this signaling pathway that can compromise pancreatic cancer.

Nodal/Activin signaling pathway

Nodal and Activin are morphogens which are being secreted into extracellular region[31,32] to mediate gene expression in target cell through phosphorylating the transcription factor mothers against decapentaplegic homolog 2, 3 and 4 (Smad2, Smad3, and Smad4)[33], and the signal intensity is found to be able to determine the cell fate decision that the target cell would execute[33]. Thus it is an important switch in deciding cell differentiation, self-renewal and pluripotency maintenance[33], the decision of the cell fate control is found to be related to the signal intensity and signal gradient generated by this pathway[33].

It is found that these two morphogens are over-expressed in pancreatic stem cells and pancreatic stellate cells, their expression levels are barely detectable in highly differentiated pancreatic cancer cell and normal pancreas or other developed tissues[34]. Moreover, it has been suggested that a small population of cancer stem cell is encompassed in pancreatic carcinomas[34], therefore, taking these two characteristics together this signaling pathway can be a specific therapeutic target for pancreatic cancer.

The common receptors of Nodal and Activin which are named Activin-like type I receptor 4 and 7 (Alk4 and Alk7, also written as Alk4/7), are being targeted by the inhibitor SB431542[34]. Targeting Alk4/7 is to abrogate the signal transduction from Nodal/Activin receptors to the transcription factors Smad 2, Smad 3, and Smad 4; and preventing the downstream genes transcription which favor tumour malignancies expression[34]. Under *in vivo* condition, pancreatic cancer cell L3.6pl pre-treated with co-administration of SB431542 and gemcitabine before implanting onto immunocompromised mice, have resulted a significant increase in apoptosis of cell carrying CD133+ surface marker, implying such regimen can deplete the population of cancer stem cell in pancreatic cancer, and prevented the tumorigenicity of the cancer cell in this xenograft model; while such observations cannot be obtained in either single treatment alone[34].

However, such regimen is challenged by the abundant stroma in the xenograft model employing primary pancreatic cancer tissue, co-administration of SB431542 and gemcitabine cannot inhibit the tumour growth in such model, overcoming the sheltering effect of stroma to the pancreatic cancer cell is vital for efficient drug delivery to the tumour cell[35]. The triple-administration of SB431542, gemcitabine and CUR199691 resulted in an enhanced depletion of cancer stem cell population, as CUR199691 is an inhibitor targets hedgehog signaling pathway and ultimately depletes the stroma[34].

Besides, pancreatic tumour cells with certain mutations on Smad 4 gene have showed to be less responsive

towards the regimen[34]. As Smad 4 is one of the factors for the signal transduction in the Notch/Activin signaling cascade[34], thus it is essential for the future studies to identify others up-stream targets controlling mutated Smad 4, so as to provide regimen for pancreatic cancer patients with mutations in Smad 4.

Nodal/Activin signaling is a promising target in elimination of pancreatic cancer stem cell from the studies presented here, despite its limitation in pancreatic cancer patients with mutations in Smad 4 gene, its effectiveness in wild type Smad 4 still makes it an attractive target in primary pancreatic cancer tissue model with the use of hedgehog inhibitor.

Metastatic suppressor-N-myc downstream-regulated gene-1

The N-myc downstream-regulated gene-1 (NDRG1) has recently been identified as a metastasis suppressor in several human cancer types[36], including human pancreatic cancer[37]. NDRG1 is found to increase the expression of tumour suppressor gene Smad4, which further inhibits the phosphatidylinositol-3 kinase (PI3K)/phosphorylated protein kinase B (AKT) signaling and extracellular signal-regulated kinase (ERK) pathway[36], besides, NDRG1 inhibits broad signaling molecules in nuclear factor - kappa B (NF-κB) signaling pathway, which resulted in reduced cancer metastasis[37]. As these three signaling pathways contribute to cancer cell proliferation and metastasis promotion, and cross-talk activities among them[36], therefore, NDRG1 is playing a role of modulator in orchestrating the signals in this triad networks.

The regulation of NDRG1 is debatable; numerous of studies have found out that hypoxia condition[38], epigenetic regulation[39] and iron depletion[40] can up-regulate NDRG1 expression level and such up-regulation seems to correlate with the differentiation status of the cancer cell.

It is worth to note that in a human PDAC model, under 2% of oxygen supply the NDRG1 mRNA and protein levels are elevated in differentiated pancreatic cancer cells but there are no change in the mRNA and protein levels in poorly differentiated cell lines[38]. Suggesting NDRG1 expression depends on both hypoxic condition and differentiation status of the tumour cell. The main focus would be on the rationale behind this phenomenon, as undifferentiated pancreatic cancer cell is comparatively more invasive and metastatic than highly differentiated counterpart[38]. In light of this, poorly differentiated pancreatic cancer cell (*e.g.*, Panc1) would have its NDRG1 level being suppressed in order to maintain high CXC chemokines[37] and high pro-angiogenic factor vascular endothelial growth factor (VEGF) expression[38] to direct cancer cell proliferation and angiogenesis. While NDRG1 over - expression has found down-regulating of these two signaling molecules and leading to suppression of tumour growth and angiogenesis[37].

The low NDRG1 expression in undifferentiated pancreatic cancer cells is related to the epigenetic regulation, as treating the undifferentiated pancreatic cancer cells with methyltransferase inhibitor 5-aza-2'-deoxycytidine has enhanced NDRG1 protein expression level, however, the epigenetic control on NDRG1 is not directly acting on the NDGR1 promoter, as there is no significant DNA methylation in the NDRG1 promoter region; suggesting other genes being silenced are essential for the NDRG1 expression[39].

As NDRG1 expression is affected by numerous factors, studies of targeting the molecular events downstream of NDRG1 are carried out, for instance an novel synthetic derivative of curcumin (CDF) has shown its inhibitory effect of the expression of VEGF, hypoxia inducible factor-1 α (HIF-1α), miR-210 and cancer stem cell self-renewal properties under hypoxia condition and are crucial for pancreatic cancer cell to promote tumour angiogenesis[41].

Although the exact mechanism of controlling the NDRG1 remains unclear, the current findings have suggested maintaining a high NDRG1 expression level in undifferentiated cell is able to suppress the tumour malignancies. Therefore, studies in finding enhancing NDRG1 expression genes is important in suppressing pancreatic cancer growth and metastasis.

Energy metabolism

As mentioned in the previous sections, the low vascularity structure of pancreatic tumour leading to a hypoxic environment and the adaptation of pancreatic cancer cells in hypoxic conditions through enhanced proliferation, angiogenesis and metastasis have been described. However, the primary element for cell survival is energy source which normally generated in glycolysis and Kreb's Cycle, as pancreatic cancer cells have an oxygen scarcity issue[42]; metabolic changes in pancreatic cancer cells allow them to cope with hypoxia.

First, the utilization of glucose would rely heavily on TCA-independent pathways, for example, there is up-regulation of pentose-phosphate pathway, anaerobic respiration for ATP production in hypoxic environment[43]. Secondly, glutamine metabolism is also elevated in hexosamine biosynthetic pathway which is crucial for the production of UDP-N-acetylglucosamine, and it is used for glycosylating proteins in proteins modification[44]. Glutaminolysis is also employed by hypoxia pancreatic cancer cell which metabolizing glutamine to generate glutamate and can be further metabolized in TCA cycle to produce pyruvate and lactate for further ATP production[44]. Lactate production is important for tumour cell invasiveness and neighboring cell proliferation, as inhibition of the enzyme glutamine fructose-6-phosphate amidotransferase by azaserine can cause significant reduction in hypoxic pancreatic cell proliferation[42]. Apart from targeting glutaminolysis, cannabinoids are found to suppress TCA cycle and induce the reactive oxygen species which leads to AMP-activated protein kinase level increase to mediate autophagy in pancreatic cancer cells[45-47]. The ROS signaling activation could also abro-

gate the electron transport chain in mitochondria with unclear mechanism[48], leading to depletion of ATP in the cell and the AMPK dependent autophagy would be mediated[45].

By considering the founding in the energy metabolism of PDAC, targeting glutamine, glucose metabolism and increase the ROS production in hypoxic region in pancreatic tumour can elicit autophagy in PDAC. It is important to evaluate the effects of targeting them in *in vivo* model, as blocking major metabolic pathways is very likely to damage normal tissues, specific targeting the metabolic pathways in PDAC would minimize such drawback and enhancing the therapeutic value of targeting the energy metabolism pathway.

RECEPTOR PROTEINS

DR5
The death receptor 5 (DR5), is found to be frequently expressed in pancreatic cancer stem cell[49] and mediates cancer cells apoptosis *via* caspase 8 recruitment upon interacting with another receptor, Tumour necrosis factor-related apoptosis-inducing ligand (Apo2L/TRAIL)[50,51], forming the death-inducing signal complex (DISC) to induce apoptosis, thus this enables the elimination of pancreatic cancer stem cell specifically, and reduces the occurrence of tumour relapse and overcoming the chemo-resistance of pancreatic cancer stem cell[52].

Several studies on targeting activation of Apo2L/TRAIL induced apoptosis have been carried out, by combing with chemotherapies to obtain a synergistic effect in shrinking cancer stem cell population in pancreatic cancer[53]. Co-administration of DR5 agonist Tigatuzumab and gemcitabine has recorded more tumour regression on PDA xenografts than administrating either agent alone[49]. Moreover the up-regulation of several signaling molecules, for instance, cell surface death receptor Fas, Fas-associated death domain, and tumour necrosis factor receptor 1-associated death domain in the apoptotic pathway are also recorded[49]. Indicating the co-administration of Tigatuzumab and gemcitabine can result in cell growth inhibition and apoptosis for cell expressing DR5[49].

Another study has showed that dihydroartemisinin can increase intracellular ROS concentration and would lead to DR5 expression elevation and in turn mediate apoptosis *via* Apo2L/topoisomerase, TNF-α-related apoptosis-inducing ligand (TRAIL)[52]. Revealing the apoptotic pathway activation through DR5 requires high intracellular ROS[52]. Therefore, eliciting apoptosis in DR5 over-expressed cancer cell is a promising therapy for pancreatic cancer[50].

PAR2
The Protease-activated Receptor-2 (PAR2) is a member of the G-protein coupled receptor family and is activated by trypsin[54]. PAR2 is able to promote angiogenesis through two distinct pathways. The first one is *via* the ac-

tivation of the mitogen-activated protein kinase (MAPK) to mediate VEGF release[55], another pathway involves the tissue factor to bind with integrin-linked kinase to up-regulate HIF-1α expression *via* AKT phophorylation and eventually enhanced VEGF expression[56]. Hence, PAR2 is essential for tumour survival under hypoxic condition in the micro environment, as PAR2 maintains a constitutive high level of HIF-1α for angiogenesis promotion and this also explains the high metastatic property of pancreatic cancer cell in hypoxia region.

Besides, the role of PAR2 in pancreatic cancer cell migration is also being reported, PAR2 is found to mediate MAPK-epidermal growth factor receptor 1/2 (EGF1/2) signaling pathway with the utilization of extracellular ATP, blocking the cross talk between PAR2 and extracellular ATP can be a target in reducing pancreatic cancer metastasis[57].

HER3
The Human Epidermal Growth Factor Receptor (HER) family consists of four members in which they are all type 1 transmembrane receptor with tyrosine kinase properties[58], except HER3[59], a member of HER which is found overexpressed 41% in pancreatic cancer[60]. Because of lacking tyrosine kinase activity in HER3, it requires phosphorylation by another HER receptor to activate PI3K/AKT signaling pathway to mediate cell angiogenesis and metastasis[61]. The expression level of HER3 has been correlated with tumour progression[59].

Therefore, HER3 has been an important target for suppressing tumour angiogenesis and metastasis by using humanized monoclonal antibodies, *e.g.* U3-1287 which has gone through phase 1 clinical trials with well tolerance in solid tumour patients , MM-121 and tyrosine kinase inhibitors[62,63]. The anti - HER3 agents block the activation site on the HER3 receptor, preventing the activation of HER3 during heterodimerization with HER2 receptor[59] and promoting receptor internalization upon binding onto its extracellular domain[58]. This reduces the activation of PI3K/AKT signaling pathway and its downstream effectors activation, resulting tumour growth suppression[59].

In view of this, because of HER3 over-expression in PDAC and its crucial role in activating the signaling pathway essential for cell growth, it is a valuable and specific therapeutic target upon co-administration of anti - HER3 agent and gemcitabine has resulted an enhanced anti-tumour effect[64], confirming the therapeutic value of anti - HER3 agents in PDAC and is worth investing in more clinical studies.

All in all, we have described three receptor proteins which carry out apoptosis, tumour proliferation and metastasis. Current studies are focusing on how to trigger the signaling molecule that could induce apoptosis, inhibit the receptors that favor cancer proliferation and metastasis, so as to reduce tumour progression. However, the possibility of combining these two approaches in the same model is not yet published, in which the

total effect on tumour clearance is expecting to be more efficient.

CELL SURFACE PROTEIN

E-Cadherin

E-Cadherin is a transmembrane protein[65] and is a member of cadherins family in which its expression in epithelial cells is controlled by intracellular signaling molecules[66]. E-Cadherin directs the positioning of the cell during morphogenesis, controlling cell migration and tissue structure maintenance[67]. It is reported that during epithelial-mesenchymal transition (EMT) the E-Cadherin level in neoplastic epithelial cells is down-regulated, suggesting triggering the dedifferentiation of neoplastic epithelial cells into mesenchymal cell with higher motility[65].

The fading E-cadherin expression is frequently reported in undifferentiated, noncohesive pancreatic cancers[68], and it is found that the silencing of E-cadherin is mediated by Snail/ histone deacetylase 1 (HDAC1)/histone deacetylase 2 (HDAC2) complex[65] and Enhancer of Zeste Homolog 2 (EZH2)[69] through hypermethylation of its promoter region[68]. Inhibition of Snail and HDAC2 are also carried out to validate the E-cadherin expression is under such complex governance[70].

Since the absence of E-Cadherin marks the onset of metastasis and PDAC progression, a study targeting E - Cadherin restoration by using microRNA 101, has inhibited the EZH2 binding on E-Cadherin promoter region in PANC1 preventing E - Cadherin silencing and in turn inhibited its tumorigenicity xenograft[69].

The key for targeting E-Cadherin to obtain therapeutic value in PDAC is to up - hold the E - Cadherin expression by down - regulating its inhibitor, as described above, inhibiting EZH2, and Snail/HDAC1/HDAC2 can reduce E-Cadherin depression and suppresses the tumorigenicity of pancreatic cancer, in the future studies, discovering targets that suppress E-Cadherin expression is important for therapy involving E - Cadherin.

Galectin-4

Galectin-4 (Gal-4) is a glycan binding proteins which belongs to the galectin family. Gal-4 is found over-expressed in cystic tumours of the human pancreas, PDAC and cancer stromal cell[71]. Activated galectins carry out several functions; include cell-cell adhesion, cell proliferation[72], mediation of intracellular signaling[73] and tumour metastasis[74], *etc.*, However the mechanisms behind are remain unknown.

A study has evaluated the inhibition effect of Gal-4 in a pancreatic cancer cell, PaTu-S cell, can lead to enhanced tumour migration[74]. The exact reason is yet to be elucidated, but it is speculated that the reduction of Gal-4 on the cell membrane would destabilize cell-cell interaction, allowing tumour cells escape[75]. Another important implication suggests Gal-4 expression may be dependent on the tumour development stage, and is vital for tumorigenesis as it promotes cell-cell adhesion[76].

Because of Gal-4 multi-roles in expressing tumour malignancies, and the little knowledge on how Gal-4 control cell migration and tumour metastasis, it is worth to investigate its related signaling pathways and identifying possible inhibitors so as to enable targeting Gal-4 in treating pancreatic cancer.

TMPRS S4

Transmembrane Protease, Serine 4 (TMPRSS4) is found highly expressed in several cancer cells, including pancreatic cancer cell[77]. However its regulation mechanism is poorly known[78], several studies have shown that TMPRSS4 can promote EMT, metastasis and invasiveness in human epithelial pancreatic[79], lung and colon cancer[77].

It is reported that EMT mediation is not solely rely on TMPRSS4 up-regulated integrin α5 to activate FAK/ERK signaling pathway and enhanced invasiveness[79] but also count on the down-regulation of E-Cadherin in TMPRSS4 up-regulated cancer cells[79].

Another downstream target of TMPRSS4 is the urokinase-type plasminogen activator (uPA) gene[80]. TMPRSS4 would activate the transcription factors of μPA *via* c-Jun N-terminal kinase mechanism before promoting μPA transcription in a cell-type dependent manner[80]. And the increased μPA gene transcription marks the increased tumour cell aggressiveness.

The coupling effects of TMPRSS4 on tumour aggravated invasiveness and metastasis with other signaling molecules (*e.g.*, integrin α5, uPA), moreover, the inverse expression pattern of TMPRSS4 and E-cadherin suggests TMPRSS4 can be suppressed by targeting E-cadherin inhibitors as previously mentioned and should be investigated in future studies. TMPRSS4 expression is affected by various signaling molecules and by considering its important role in expressing tumours malignancies, it is a target with multiple approaches for suppression.

IAP

Inhibitor of apoptosis protein (IAP) is a group of proteins bind to caspases and inhibit caspases apoptotic effect resulting apoptosis abortion[81]. The importance of apoptosis mediation in cancer therapies has an irreplaceable place, and therapies incapable to induce cell death would be meaningless. However, most therapies nowadays involve the elicitation of apoptosis at their end, and resistance of the corresponding therapies developed due to the presence of IAP[82]. Therefore IAP is the obstacle to tackle with, so as to ameliorate the effectiveness of therapies targeting apoptosis induction.

Two of the IAP members would be discussed here which are X-linked IAP (XIAP) and survivin, because of the reports of their close interaction in triggering anti-apoptotic effect[82].

Survivin's action has been controversial in anti-apoptotic activity[82], it is reported that survivin carries out neurogenesis, angiogenesis, cell cycle progression in cancer cell[83] and displays caspase inhibitory effect through associating with XIAP and stabilizes XIAP *via* their bac-

ulovirus-inhibitor of apoptosis repeat (BIR) domain[84]. Most of the survivin inhibitors that are under clinical trials have improved the effectiveness of chemotherapies *e.g.*, topoisomerase, TRAIL[82]. Revealing survivin's submissive and supporting role in IAP targeted treatment.

XIAP is the most studied IAP, it is found able to suppress caspase-3, caspase-7 and caspase-9 apoptotic activities[85]. Inhibition of its caspase binding domains, which are named, BIR-2 and BIR-3, with the use of phenylurea-based chemical inhibitors of XIAP (XAntags) could make pancreatic cancer cells more vulnerable to apoptosis[85].

Because of the anti-tumour effect in inhibiting XIAP and the supportive role of surviving in apoptotic inhibition, co - suppressing XIAP and survivin has also been performed in Panc-1 cell[86], resulting cell proliferation hindrance, and enhanced gemcitabine effectiveness in XIAP and survivin suppressed model than sole suppression of either IAP[86].

Nevertheless, there is no cell toxicity recorded in XIAP knocked out mouse model and *in vitro* cell model, possibly by the compensatory up-regulation of other cIAPs, and the masking effect of such up - regulation requires further studies[81]. From the recent findings in IAP, inhibiting IAP is a promising therapeutic direction in promoting apoptosis progression in PDAC cells, thus enhancing the effectiveness of current chemotherapies upon co - administration in treating PDAC.

NON-CODING RNA

MicroRNA

MicroRNAs (miRNAs) consist of 18-24 base pair which are small and non-coding-sequence[87]. They execute target gene expression control by binding miRNA 3'UTR on to the target gene mRNA[87], and only when perfect binding of miRNA on to the target mRNA could mediate mRNA cleavage, otherwise, it would result into inhibited protein production[88]. miRNAs which induce over-expression of oncogenes are termed the oncogenic miRs (onco-miRs), on the other hand, miRNAs which suppress cell transformation are named tumour suppressor miRs (TSG-miRs)[89]. The abnormal expression levels of these two kinds of miRNAs are observed in pancreatic cancer[90]. Current studies are either suppressing onco - miRs or reconstituting the TSG-miRs level[91], therefore, in the following we will discuss some TSG-miRs which are promising therapeutic targets in pancreatic cancer.

miR-34: miR-34 is reported to be up-regulated by p53[92], inducing cell cycle arrest in primary and tumour derived cell lines[93]. A significant reduction of miR - 34 expression level in gastric cancer cells with p53 mutation has been observed and reconstituted miR - 34 expression by transfecting pancreatic cancer cells with letivirus carrying vector expressing miR - 34[94], and resulted in decreased Notch2 and Bcl-2 protein production, reduced tumour-sphere formation from cancer stem cell (CSC)[94]. Al-

though the relationship between miR-34 and p53 is still unclear[94], the encouraging results generated by miR-4 in p53 deficient pancreatic cancer cells[93] have make it a worthy therapeutic target.

miR-143: miR-143 has been studied for its anti-metastasis and anti-tumour proliferation in liver undergone metastasis and a pancreatic cancer xenograft in mouse model, respectively[95]. miR-143 expression level in KRAS mutant pancreatic cancer cells is also being ablated[96], re-expressing miR-143 in its deficit cell lines has performed, GEF, RAC1, matrix metalloproteases (MMPs) and KRAS are the inhibition targets for miR-143[95], as described previously lessened RAC1 level can inhibit metastasis and tumorigenesis, while inhibiting KRAS is even more important, which implies a board spectrum of signaling pathways diminishing effect.

Another tumour growth promotion factor that miR-143 targets is the cyclooxygenase (COX-2)[97], COX-2 is reported as an essential factor for prostaglandin synthesis to mediate inflammation and cancer cell growth and survival[98]. In pancreatic cancer cell, miR - 143 was found to be repressed by prostaglandin[99], and restoration of miR-143 level can decrease both mRNA and protein level of COX-2 and inhibited cell growth[98].

miR-200: miR-200 is a family of miRNAs related to EMT[100], reconstituted expression level of miR-200 has restored the phosphatase and tensin homolog (PTEN) expression level[100], as PTEN is widely down regulated in various cancer cell lines and is a tumour suppressor gene in which reduced expression would lead to enhanced tumour aggressiveness[101,102], while membrane type-1-matrix metalloproteinase (MT1-MMP) is up-regulated and lead to aggravated cancer invasion[101-103]. Restoration of miR-200 by using CDF, which is a synthetic analog of curcumin, and a natural compound, BR - DIM are reported and are found able to enhance PTEN expression level and a decreased MT1-MPP promoted invasiveness[100]. Therefore, agents which could enhance miR-200 expression would have promising therapeutic value in curbing pancreatic cancer aggressiveness for enhanced treatments efficiency.

The three TSG-miRs exemplified the diverse roles of miRNAs in anti-tumour activities, up-regulation of TSG-miRs can suppress tumour malignancies expression, however, suppressing onco-miRs that can up-regulate oncogenes also have tumour malignancies suppression effect, therefore screening and studying the agents that can up-regulate TSG-miRs and down-regulate onco-miRs are vital for PDAC therapy development.

Long non-coding RNA

Long non-coding RNAs (lncRNAs) are transcribed from intergenic and intronic regions in human genome[104] by RNA polymerase II[105], which lengths more than 200 bp[106] and their biological functions have been reported, for instance, epigenetic control, transcription regulation, pre and post-translational regulation[107], cell cycle and

differentiation control and even governing the apoptosis process[108]. lncRNA is used as a diagnostic parameter and can be a therapeutic target in cancers[104]. However, the definition and discovery of lncRNAs are expected to keep on changing as very little is known in this emerging area[109]. In the following, two lncRNAs that are highly expressed in pancreatic cancer will be discussed.

HOTAIR: HOX transcript antisense RNA (HOTAIR) is a lncRNA which is highly expressed in a range of primary tumours and metastatic cell[110], in which its expression pattern is variable but in general is also over-expressed pancreatic cancer[111]. HOTAIR carries out tumour supporting effect by inhibiting anti - tumour genes activity, in which the interaction of HOTAIR and a Polycomb-group (PcG) family protein named, EZH2, would promote chromosomal histone protein histone 3 protein at lysine 27 (H3K27) trimethylation, which leads to repressed transcription of multiple gene targets[112]. However, there are some genes inhibited in an EZH2-independent manner[113].

Suppressed HOTAIR expression by using RNAi in pancreatic cancer cell has caused retarded cell growth, diminished tumour aggressiveness; altered cell-cycle progression and apoptosis induction[114]. Thus, relieving the repressed transcription of the tumour suppressor genes by suppressing HOTAIR expression is therapeutically valuable in treating PDAC. As the studies of genes activation mechanism and the genes that are targeted by HOTAIR are still ongoing[114], and the mechanism of genes being independently regulated by EZH2 but dependent on HOTAIR only, are currently under studies.

Studies of targeting HOTAIR in PDAC cell lines have achieved reduced tumour malignancies expression, indicating the relieved tumour suppressor genes expression by targeting HOTAIR has made HOTAIR an attractive target in pancreatic cancer therapies development. However, cautions should be taken on the over-expressed genes induced by HOTAIR, as HOTAIR induced and suppressed multiple genes at the same time and some of the over-expressed genes expression level do not reduce with HOTAIR suppression[114], suggesting another mechanism may exist in down-regulating them. In conclusion, a more thorough understanding on the regulation and the functions of HOTAIR induced and suppressed genes, it could lead to a more rounded target in promoting tumour suppressors genes functions while inhibiting oncogenes activities.

MALAT1: Metastasis-Associated Lung Adenocarcinoma Transcript 1 (MALAT1), as known as the Nuclear-Enriched Abundant Transcript 2[115] is found highly expressed in normal pancreatic and lung tissues with high abundance and highly conserved among mammalian[116]. Intensive studies of MALAT1 in non-small-cell lung carcinoma revealed its metastasis and tumorigenicity promotion activities[117]. Although inadequate studies of MALAT1 in pancreatic cancer cell model, it has been reported for its promotion of tumour malignancies expression in various cancer types[116]. For instance, in colorectal cancer, a Chinese herb extract Resveratrol is shown to down-regulate MALAT1 and cause suppression of Wnt/β signaling *via* decreasing β-catenin nuclear localization and eventually inhibited the invasiveness and metastasis of colorectal cancer[118].

Apart from promoting tumourigenesis, invasiveness and metastasis, MALAT1 also participates in the control of cell cycle progression, oncogenic transcription factor B-MYB is found up-regulated with the expression of MALAT1, silencing of B-MYB in fibroblast model resulted into cell cycle arrest in G1/S and S phase[119], moreover, another transcription factor, E2F transcription factor 1 (E2F1), which is essential for cell cycle progression and is also under the modulation of MALAT1, however down - regulated MALAT1 brought down E2F1 expression has elicited p53 expression enhancement and lead to cell cycle arrest and hence reduced cell proliferation[119]. This implies MALAT1 could induce DNA damage response *via* an unknown mechanism[119].

With regard to the findings of MALAT1 in other cancer, MALAT1 expression in PDAC is also very likely to correlate to pancreatic cancer progression. Although MALAT1 expression level is high in normal pancreatic tissue, its expression level in pancreatic cancer is not yet reported and also the role of MALAT1 in pancreatic tumour activities. Thus, if MALAT1 has a similar tumour malignancies promotion role in pancreatic cancer as in other cancer types, it would be a promising therapeutic target for PDAC treatment development.

EPIGENETIC MODIFIER

HDAC

Histone Deacetylases (HDACs) are a group of four classes of deacetylases[120], each class of the enzyme contributes to different tumour malignancies expression, for example, as mentioned previously, HDAC1 is responsible for the acceleration of EMT and metastasis in PDAC[121], while HDAC2 would desensitize the PDAC towards DNA damage response and decreased prop - apoptotic proteins[122], however, only the third class, which is named the human hst proteins (SIRTs) did not respond to HDAC inhibitors (HDACIs) under current clinical trials[120], but a HDACI named Sirtinol is able to induce apoptosis with the administration of Sirtinol[121], and its effect is further enhanced with the co - administration with gemcitabine[123].

The exact mechanism of HDACIs in mediating anti-tumour activities remain further elucidation. However, studies have shown that it is not necessary for HDACIs to inhibit the expression of HDACs in mediating tumour suppression, for instance, a class I and II HDACI did not cause changes in the expression level of HDAC1, and other tumour suppressor genes but has shown reduced cell proliferation in cervical tumour cell[124]. Moreover, a class I and II HDACI inhibitor is found able to cause

a changes in the expression profile of class III HDAC, SIRTs[121]. These evidences suggest the working mechanism of HDACIs involve complex molecular control. On the other hand, Rel/p65 (NF-κB) is found relating to the expression level of HDAC, for instance, over-expression of class I HDAC in pancreatic cancer cells, their expression level of NF-κB is also high, besides, a class I HDACI valproic acid (VPA) is studied and found to cause a decreased expression in pancreatic cancer cells which leads to enhanced PDAC apoptosis[125], in which over-expression of NF-κB has been reported for enhanced tumour growth, angiogenesis, chemo-resistance and metastasis[125]. Blocking NF-κB activity by VPA can obtain anti-tumour effect in such case[126].

The action of HDAC on gene silencing is mediated by deacetylating the histone proteins in the chromatin leading to chromatin condensation, resulting silenced genes transcription[121]. As the genes being silenced in cancer are mostly related to tumour suppressors, anti - apoptosis, and often resulted in drug resistance, therefore, targeting HDAC by using HDACI is believed to reduce the these tumour malignancies expression by suppressing the related signaling pathways of the PDAC and synergistically enhance the anti - tumour effect of current chemotherapy.

BMI1: B-Cell-specific Moloney murine leukemia virus Insertion site 1 (BMI1), belongs to the polycomb group (PcG) which represses transcriptional activity of various genes[127]. Over-expression of BMI1 in a board spectrum of cancer cells is observed, it strengthens tumour growth by providing anti-apoptotic activities and participate in tumour metastasis by up-regulating PI3K/AKT signaling pathway[127]. In *in vitro* experiment, PDAC with BMI1 suppressed using shRNA has shown enhanced cell death in response to gemcitabine treatment, a significant decrease for its cell surface markers $CD44^+CD24^+ESA^+$, loss of self-renewal ability, reduced tumour sphere formation by CSCs and reduced tumour size in xenograft model[127].

Because of the diversified anti-tumour effects of silencing BMI1 in pancreatic cancer cell, such as reduced invasiveness, tumourigenesis[127], metastasis, CSC phenotypes, cell proliferation[128] and also chemo-resistance[127]. Besides, CSC is reported to be the causes for tumour relapse in pancreatic cancer[129], thus, the diversified roles of BMI1 in pancreatic cancer have made it a very attractive target, future studies targeting BMI1 inhibition and its downstream effectors would benefit PDAC treatment development.

EZH2

The polycomb repressor complex 2 member, Enhancer of Zeste Homolog 2 (EZH2) is a histone methyltransferase which is highly expressed in pancreatic cancer cells[130], EZH2 mediates tumour suppressor genes transcription inhibition through trimethylation of the H3K27[131], such as suppressing Rap1GAP expression in squamous car-

cinoma[132], E-Cadherin in pancreatic cancer[131]. Besides, several reports have suggested EZH2 suppresses miRNAs in contributing to pancreatic cancer progression, *e.g.*, microRNA-218 (miR-218), microRNA-26a[133,134], miR-218 is essential in suppressing tumour proliferation and metastasis in nude mouse model[133], EZH2 is believed to interact with two polycomb repressive complexes (PRCs), PRC1 and PRC2, and promoting the methylation of the target miRNA promoter region to silence the miRNAs expression in pancreatic cancer[133]. It is found that with the administration of EZH2 inhibitor, such as 3-deazaneplanocin A (DZNeP), can reduce EZH2 expression of EZH2 and rescued the expression of miR-218 leading to tumour malignancies reduction[133,135].

Apart from suppressing miRNAs in tumour progression, studies of the role of EZH2 as a tumourigenesis initiator have found that EZH2 also suppresses tumour suppressor gene $p16^{INK4}$, in which it suppresses tumour proliferation and regeneration, enhanced EZH2 expression has caused $p16^{INK4}$ down - regulation, counteracting the suppression effects exerted by $p16^{INK4}$[136]. Such control is crucial for the regeneration of the injured acinar pancreatic cell, in which the injured cell undergone de-differentiation into metaplastic epithelial intermediate, depleted $p16^{INK4}$ allows the cell to re-differentiate into acinar cell from metaplastic epithelial intermediate[136]. Thus in combination with the early appearance of PaIN lesion in pancreatic cell baring KRAS mutation and the loss of $p16^{INK4}$ expression due to enhanced EZH2, invasive and metastatic tumour development are accelerated, demonstrating the linkage between regeneration and tumourigenesis under the influence of mutant KRAS[137].

Further studies of the role of EZH2 in pancreatic CSC has found it is essential in maintaining the CSC population in pancreatic cancer, suppressing EZH2 has decreased the degree of H3K27 methylation, reduced CSC population in pancreatic cancer, enhanced genes expressions for cell differentiation and migration[130]. Since the trimethylation of H3K27 and the expression is correlated with the CSC population, it is suggested that the H3K27 trimethylation by EZH2 can be used as a marker for the CSC population which allows rapid evaluation for the population of CSC when compared to conventional methods, hence, speeding up the studies of the effectiveness of compounds towards pancreatic CSC[130].

From the current findings of suppressing EZH2 in pancreatic cancer, EZH2 has an important role in tumour development initiation and supporting cancer stemness, and co-administration of DZNeP and gemcitabine has achieved promising anti-tumour effects. Nevertheless, EZH2 has also demonstrated its possibility to act as an indicator for CSC population estimation, and CSC elimination is an important factor for researchers to evaluate the efficacy of the compounds under studies, thus EZH2 is a versatile targets that possess both therapeutic and assay values and screening compounds suppressing EZH2 would definitely help speeding up therapies development

in PDAC.

PANCREATIC CANCER STEM CELL (PANCREATIC CSC)

The tumour cell population is reported to encompass a population of cancer stem cell (CSC)[138], and it is reported to give rise to the cancer stemness in various cancers, by carrying out self-renewal, metastasis, invasiveness enhancement[139], and drug resistance for pancreatic tumour[140]. Studies in CSC have led to the discovery of distinguishable cell surface markers presented in various cancer types, and this allowed the isolation of cancer stem cell for various studies[141]. In this section, we will briefly discuss how CSC contributes to enhanced cancer malignancies, and the plausible targets in CSC that have been reported to have therapeutic value.

Signaling pathways in CSC

There are three members of hedgehog proteins in the hedgehog signaling[141], a member of the hedgehog family, sonic hedgehog is found over - expressed in both pancreatic cancer cell and CSC[142]. The up-regulated sonic hedgehog signaling molecules facilitates the development of PanIN and enhanced accumulation of mutations in KRAS while inhibiting the hedgehog signaling pathway by the hedgehog signaling inhibitor cyclopamine has resulted decelerated tumour growth and on set of apoptosis[142], another inhibitor GDC-0449 is reported to produce reduced cell viability, caspase-3 mediated apoptosis, reduced tumour sphere formation in pancreatic CSC[143]. Sonic hedgehog has displayed its critical role in tumourigenesis initiation and tumour proliferation, targeting hedgehog signaling is therefore advantageous in the early development of pancreatic cancer.

In Notch signaling pathway, over-expressed Notch-1 promotes EMT and tumour sphere formation[144], which is confirmed by the increase expression of CD44 and EpCAM cell surface markers on CSC[144], suggesting Notch as a factor in pancreatic tumourigenesis in CSC, but the role of it in CSC self-renewal requires further studies[141]. Notch mediates signaling by nuclear translocation and is modified by γ-secretase before entering the nucleus, thus inhibitors of γ-secretase have been used to study the role of Notch in pancreatic cancer and also pancreatic CSC[145], a Notch inhibitor, PF-03084014 is found able to bring a reduction of CSC population, tumour re-growth and inhibited several cancer malignancies, *e.g.*, tumour growth, angiogenesis in pancreatic cancer xenograft model with the co-administration with gemcitabine[146], therefore its effect on pancreatic cancer is expectable.

The CXCR4 signaling pathway which comprises the ligand, stromal cell - derived factor-1/CXCR chemokine ligand 12 (SDF-1/CXCL12) and the G-protein coupled receptor, CXCR4. This signaling pathway is up - regulated in pancreatic cancer cell due to enhanced expression of CXCR4, and resulting into tumour metastasis promo-

tion; enhanced migration and strengthened stromal adhesion[141]. In pancreatic CSC, co-expression of CD133+ and CXCR4+ on the CSC signified a highly metastatic characteristic and contributes to tumour metastasis, therefore, disrupting the SDF-1 mediated CXCR4 signaling and depletion of the CD133+ CSC can abrogate the metastatic characteristic of pancreatic tumour[146]. Although targeting the CXCR4 in stopping pancreatic tumour metastasis looks promising, CXCR4 inhibitors are found highly toxic and non - specific reaction are the drawbacks that must have to be overcome before translating into clinical trials or practices[147].

Forkhead Box M1 (FoxM1) is a transcription factor found over-expressed in pancreatic cancer in which it promotes the expression of EMT characteristic[141], which is deduced by the increased mesenchymal cell markers expression including, zinc-finger E-box binding homeobox 1 (ZEB1), ZEB2, E-cadherin, and vimentin, and also enhanced tumour sphere formation which marks the strengthened self-renewal ability for CSC[148], as enhanced EMT is having close resemblance to CSC in giving rise to cancer stemness[149]. A natural compound genistein can inhibit the FoxM1 signaling pathway by down-regulating the expression of FoxM1 and its downstream gene targets (*e.g.*, VEGF, MMP-9) leading to reduced EMT and reduced tumour sphere formation and have resulted into reduced tumour growth and enhanced apoptosis[150]. The exact mechanism of the regulation of genistein on FoxM1 and its target genes is not clear yet, however, the application genistein can rescue the microRNA-200 (miR-200) expression by attenuated FoxM1 expression, and enhanced expression of miR-200 can inhibit the EMT progression[147]. Because of the important role for FoxM1 plays in the EMT and CSC progression, and the availability of FoxM1 inhibitor has made FoxM1 an attractive target and should evaluate the anti - tumour effects under co-administration of genistein and gemcitabine.

Cell surface marker on CSC

There are several cell surface markers on CSC which are not only be used to isolate CSC, but also have important functions towards CSC. For instance, expression of CD44+/CD24+/ESA+ mark the pancreatic cancer cell that function as CSC, with several signaling pathways (*e.g.*, BMI1, sonic hedgehog) up-regulated and self-renewal and tumorigenesis enhancement are observed[151], while ablated CD133 would lead to loss of CSC self - renewal ability[146]. This demonstrates the markers presented on the CSC can provide some clue on the de-regulated signaling pathways in the tumour which can help deciding the targets of the sub-population of the pancreatic tumour. Nevertheless, a novel CSC marker, c-Met, is found to be essential for tumour growth, tumour sphere formation and metastasis, inhibiting the expression of c-Met have suppressed these tumour malignancies[152], possibly *via* the downstream signaling pathways of c-Met, such as Ras-MAPK, and PI3K-AKT[153]. With the emerging knowl-

edge of the cell surface markers and their downstream signaling, options for targeting signaling transduction in PDAC is ever growing, besides, the surface markers can also act as the reference reflecting the de-regulated signaling pathways, hence, facilitating the therapeutic direction formulation.

TUMOUR MICROENVIRONMENT

Matrix metalloproteinase

MMPs are a group of zinc-dependent endopeptidases which hypothetically can degrade almost all proteins in the extracellular matrix (ECM)[154]. MPPs are over-expressed in pancreatic cancer and the biological roles of MMPs in cancer are to digest the proteins in basement membrane in the ECM which leads to enhanced migration of tumour cell[155], an evidence of migration signal mediation by cleavage of laminin-5 in ECM has been reported[156]. Moreover, cleavage of E-Cadherin by MMP-3, MMP-7[157] and A disintegrin and metalloprotease 10 (ADAM10) are observed, the loss of E-Cadherin not only enhanced cell mobility but also enhanced the tumour invasiveness and migration[158]. In addition, the release of pro-angiogenic inflammatory cytokine (TNF-α)[157] and VEGF[159] are correlated with the MPPs activity and all these confirm the role of MPPs in EMT and tumour metastasis promotion[160].

MMP is found related to the pancreatic stellate cell, which will be described in the next section, the TGF-α up-regulation in pancreatic stellate cell correlates with up-regulated MMP-1, suggesting a possibility of the association of these two molecules overexpression in enhanced tumour cell invasion[161].

As the importance of MMPs in tumour angiogenesis and metastasis is undeniable, studies of formulating MMP inhibitors (MMPIs) are undergoing; a MMPI, SB-3CT is able to reconvert the MMP-2 into its pro-enzyme state and has brought down liver metastasis[162]. However due to the usual late stage discovery of pancreatic cancer in real life[163] the use of MMPIs is limited and also MMPs activities have been observed to be stage dependent[164], therefore MMPs can be targeted for PDAC patients with early detection and can be applied widely when early detection method for PDAC is developed.

Pancreatic stellate cell

Pancreatic stellate cell (PaSC) resides in the exocrine of the pancreas and has dual roles in normal pancreatic tissue[165], first it acts as a storage of vitamin A[166], secondly upon pancreatic injury, the PaSC would be activated to acquire a myo-fibroblast-like phenotype which is called activated PaSC[167-169], activated PaSC will secret proteins into the ECM[170] resulting into pancreatic fibrosis[165] and on setting chronic pancreatitis which could lead to high risk of PDAC development. As mentioned in 10.1, high TGF-α expression level correlates with the high MMP-1 expression level in inducing PaSC migration, inhibition of MMP-1 has showed such migration induction is curbed by using MMP-1 tissue inhibitor and siRNA of

MMP-1[161], indicating PaSC activity can be modulated *via* MMPs.

Other studies by targeting PaSC proliferation and migration have been carried out, transgelin has been reported to be over-expressed in activated PaSC but not in normal acinar cell which could cause pancreatic fibrosis[171]. Moreover, knocking down transgelin expression has reduced cell proliferation and migration abilities in *in vitro* experiment[171], providing a biomarker for specific therapeutic target in knocking down PaSC population in the future.

Hedgehog signaling pathway

The Hedgehog (hh) signaling pathway, involves the secreted signaling molecules hedgehog proteins, which is classified into 3 subcategories, namely, Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh)[172]. Among these 3 hh, Shh is found over-expressed in 70% of primary PDAC[173]. Hedgehog biological roles have been described as an essential factor in embryonic development and regulate cell proliferation[172].

The mediation of hedgehog signaling is triggered upon the binding of Shh to the Patched 12-transmembrane domain receptor (Ptch) which further activates another transmembrane signal transducer, smoothened (Smo), that would lead to localization of transcription factors in the nucleus and initiate transcription of downstream effectors[174], for instance, Cyclin D2, FoxM1, jagged 2 (JAG2), *etc*[175].

It is reported that tumourigenesis and tumour proliferation requires constitutive activated hedgehog signaling, and in pancreatic stromal cell in PDAC, Smo is over - expressed and direct tumour cell growth in the vicinity of stromal cell, leading to a therapy targeting hedgehog signaling in tumour-stromal interaction[172]. Besides, report of Shh activation in CSC is crucial for CSC proliferation[176], and it has been discussed for the CSC in aggravating pancreatic cancer treatment, such as heightened drug resistance and tumour relapse. Therefore, studying hedgehog signaling inhibitors is beneficial for pancreatic cancer treatments. A hh signaling inhibitor, Sulforaphane has been found to inhibit self-renewal capacity in CSC *via* Shh signaling inhibition leading to downstream effectors *e.g.*, Nanog and Oct-4 suppression[176]. Moreover, inhibition of hedgehog in pancreatic cancer cells and tissue are performed and it is found that a marked decrease in EMT with EMT related transcription factors (Snail and Slug) down - regulation and had suppressed PI3K/AKT signaling, which is downstream of hedgehog signaling with an association of decreased cell proliferation[177].

Because of diversified roles of hedgehog signaling in tumour malignancies and CSC population maintenance, and the cross talk among other signaling pathways, *e.g.* FoxM1, Notch (*via* JAG2), targeting hedgehog may have a centralized effect in weakening the malignancies of pancreatic tumour.

Stromal environment-hyaluronan

The microenvironment of pancreatic cancer, has been

accused to be the major challenge in drug delivery because of its highly dense ECM, the penetration of even small drug molecules gemcitabine is prevented[178]. Besides, stromal cells which are the activated fibroblasts and PaSC, inflammatory cells[178]; and distorted vascular structure of blood and lymphatic vessel composed the stromal environment[179]. And the production of stroma is mediated by various factors involved in numerous signaling pathways in an autocrine and paracrine action, TGF-β, insulin growth factor 1, and EGF are the examples[180].

Among the molecules in ECM, hyaluronan or hyaluronic acid (HA) is secreted by PDAC[181], and is a repeat of N-acetylglucosamin/glucuronic acid disaccharide[170]. It is able to interact with a hyaladherin, CD44, to regulate tyrosine kinase receptor and to facilitate angiogenesis, EMT, and chemoresistance[182]. It is also one of the main components that contributes to high intra-tumoral fluidic pressure (IFP) through solvating with water molecule, hence, impeded the diffusion of drug molecules into the target tumour cell[183]. It is found that co-administration of hyaluronidase with gemcitabine or other drugs prolonged the localization of the accompanied drug in the tumour[183]. Therefore, it would become a trend for future development of drug target, *e.g.*, well incorporated with hyaluronidase to facilitate drug delivery, or even HA can be a target to disintegrate the condensed ECM, enhancing the responsiveness of the tumour cell to the treatment.

CONCLUSION

A range of therapeutic targets in PDAC have been briefly described in this article, in which their anti - tumour and oncogenic activities are characterized through various experiments and can be taken as potential target for PDAC therapies development.

Nevertheless, numerous of the targets are found overlapped with each other in producing certain kinds of tumour malignancies, *e.g.*, over-expression of CXCR4, Rac1, BMI1, and *etc.*, in pancreatic tumour cell have observed a metastasis enhancement. In light of this, and hypothetically, in order to prevent metastasis, suppressing these targets should have a more pronounced effect in metastasis inhibition. Moreover, such outflanked approach may also prevent the tumour cell from switching into other signaling pathways producing the same tumour malignancies, and achieving elimination ultimately. Besides, the current knowledge of each of these targets is insufficient, categorizing these targets by the tumour malignancies produced and identify if there is any relationship between them and understand the mechanism behind, would allow the discovery of linkages among them in terms of proteins and mRNA expression levels and mechanistic studies.

Last but not least, screening of suitable inhibitors for these targets is crucial in putting these targets into practice. Toxicity of some of the inhibitors mentioned is reported, while, traditional Chinese medicine (TCM) may be a good source for screening inhibitors that are less or non-toxic compounds, *e.g.*, an EZH2 inhibitor, davidian, is extracted from TCM *Polygonum capitatum* without toxicity observed in xenograft model[184].

Single effort from one side is far from enough in pancreatic tumour elimination due to its high malignancy and complex tumour microenvironment, multiple targets have to be considered in developing PDAC therapies, therefore, the way of applying these targets and which targets should be applied require further effort.

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2014 ADVANCES IN PANCREATIC CANCER

Therapeutic options for the management of pancreatic cancer

Maria L Rossi, Azeem A Rehman, Christopher S Gondi

Maria L Rossi, Azeem A Rehman, Christopher S Gondi, Department of Medicine, University of Illinois College of Medicine-Peoria, Peoria, IL 61656-1649, United States

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Correspondence to: Christopher S Gondi, PhD, Assistant Professor, Department of Medicine, University of Illinois College of Medicine-Peoria, Box 1649, Peoria, IL 61656-1649, United States. gondi@uic.edu

Telephone: +1-309-4958167 Fax: +1-309-6557732
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Abstract

Since its initial characterization, pancreatic ductal adenocarcinoma has remained one of the most devastating and difficult cancers to treat. Pancreatic cancer is the fourth leading cause of death in the United States, resulting in an estimated 38460 deaths annually. With few screening tools available to detect this disease at an early stage, 94% of patients will die within five years of diagnosis. Despite decades of research that have led to a better understanding of the molecular and cellular signaling pathways in pancreatic cancer cells, few effective therapies have been developed to target these pathways. Other treatment options have included more sophisticated pancreatic cancer surgeries and combination therapies. While outcomes have improved modestly for these patients, more effective treatments are desperately needed. One of the greatest challenges in the future of treating this malignancy will be to develop therapies that target the tumor microenvironment and surrounding pancreatic cancer stem cells in addition to pancreatic cancer cells. Recent advances in targeting pancreatic stellate cells and the stroma have encouraged researchers to shift their focus to the role of desmoplasia in pancreatic cancer pathobiology in the hopes of developing newer-generation therapies. By combining novel agents with current cytotoxic chemotherapies and radiation therapy and personalizing them to each patient based on specific biomarkers, the goal of prolonging a patient's life could be achieved. Here we review the most effective therapies that have been used for the treatment of pancreatic cancer and discuss the future potential of therapeutic options.

Core tip: Pancreatic ductal adenocarcinoma has challenged researchers for decades. It remains one of the most deadly cancers due to the complex molecular and genetic makeup of its cancer cells and their surrounding microenvironment. In addition, there are no valid screening tests available to detect pancreatic cancer in its early stages. Yet, as knowledge of this cancer has evolved over time, so have novel methods for treating it. Researchers have a deeper understanding of pancreatic cancer now than ever before. The future holds much promise for new breakthroughs that will significantly improve patient outcomes.

INTRODUCTION

Despite the improved survival rates noted in numerous cancers, including breast[1-3], prostate[4] and colon cancer[5], the overall survival rates for patients diagnosed with pancreatic cancer have shown little improvement over the past thirty years[6-8]. Pancreatic ductal adenocarcinoma (PDA) remains one of the most rapidly progressive and deadly malignancies worldwide[4]. The prevention of pancreatic cancer is difficult to assess, due to limited studies identifying potential risk factors compounded with the multifactorial, heterogeneous nature of the disease. Cigarette smoking has been noted to double the risk of pancreatic cancer, yet only accounts for 20%-25% of the cases[9,10]. Additionally, family history may also contribute a significant role as 5%-10% of individuals with pancreatic cancer report an incidence of pancreatic cancer in a close family member[11]. This risk is further substantiated when there is a larger number of family members with pancreatic cancer and a decrease in age of onset in kindred[12]. Other noted risk factors include alcohol abuse[13], a high-fat diet[14,15], and certain trace elements[16].

The challenge of diagnosing PDA at an early stage further contributes to the dismal five-year survival rate that is projected for patients. Located in the retroperitoneum of patients who present with non-specific symptoms, PDA is not diagnosed until it has reached an advanced clinical stage in 80% of patients[17]. In addition, lack of effective screening and early biomarker detection methods have prevented clinicians from identifying this cancer in a pre-malignant stage. Ideally, visual evaluation *via* computerized tomography (CT) and magnetic resonance imaging (MRI) should be incorporated upon suspicion of pancreatic cancer for detection and resectability assessment[18]. Although CT scan has often been utilized to detect pancreatic cancer[19-21], reliance on MRI, particularly in regard to assessing local invasion and metastasis, has increased[22]. Other imaging may also provide certain benefits, such as endoscopic ultrasound for investigating vascular invasion[23], fludeoxyglucose-positron emission tomography scanning for recurrent tumors[24], and laparoscopy for more accurate staging[25]. While the use of these techniques remains helpful to determine prognosis and treatment regimen for patients diagnosed with pancreatic cancer, none have been validated as effective screening tests for general or high risk populations.

Once diagnosed, a number of chemotherapy, radiation and combination therapy regimens have been used to treat patients with ductal pancreatic tumors. Unfortunately, the dynamic molecular and cellular makeup of individual pancreatic tumors, renders many of them resistant to the majority of these therapies. Although surgical resection has been shown to increase patient survival by 10 mo[26], the majority of patients who undergo these procedures experience comorbidities and recurrence. Current research has identified additional sources of therapeutic resistance in the microenvironment of these tumors. Characterized by stromal proliferation, reduced angiogenesis and a unique subset of cells known as cancer stem cells (CSCs), the tumor microenvironment has become a target of new therapeutic agents.

While improved understanding of pancreatic cancer biology has lead to several therapeutic breakthroughs in the treatment of PDA, major progress toward improving survival rates in patients has been extremely slow. However, as our understanding of this tumor's therapeutic resistant nature improves, so will future progress in treating pancreatic cancer.

CLINICAL PRESENTATION AND DIAGNOSIS

One of the greatest challenges in treating pancreatic ductal adenocarcinoma (PDA) is discovering it in the pre-malignant stage. The average patient diagnosed with pancreatic cancer is in their seventh decade of life and presents to their primary care physician with general symptoms of abdominal pain and weight loss[27]. Not only is the pancreas difficult to palpate due to its retroperitoneal location, but there are also no specific blood tests to confirm suspicion of malignancy. More specific symptoms, such as unexplained jaundice[28], onset of diabetes[29] and development of thromboembolic disease[30] are more diagnostic of pancreatic cancer, but do not present until later stages of the disease. The primary comorbidities associated with PDA include biliary obstruction, infection, ascites, pancreatic insufficiency and in advanced stages of the disease, cachexia[31]. Unfortunately, once a patient presents with these symptoms, the disease has often already reached its malignant stage and the patient may never be able to receive treatment.

Effective screening tests to provide early diagnosis of pancreatic cancer could potentially prevent these symptoms. The ones that do exist are not validated. For example, although endoscopic ultrasounds provide a higher yield of detecting pancreatic cancer in its early stages, the comorbidities associated with this procedure render it an unsuitable screening test in the general population. As a result, studies are currently underway to identify high risk individuals who may benefit from this invasive procedure[32-34]. Other techniques, such as cross-sectional imaging could be used to identify asymptomatic pancreatic neoplasms for surgical resection[34] as long as they are confirmed by CT or MRI techniques which provide better resolution between normal and neoplastic pancreatic tissue[35].

Although a greater understanding of the molecular events in pancreatic cancer tumorogenesis has lead to the discovery of biomarkers that help to predict the tumor's response to treatment, there has been no use of these markers in cancer drug development[36]. The only biomarker

Table 1 Cellular mechanisms of therapeutic resistance in pancreatic cancer

Cellular pathways	Mutated gene	Ref.
Cell-cycle control	*CDK2NA* (90%); *APC2*	Almoguera *et al*[54]; Schutte *et al*[71]; Hahn *et al*[72]
RAS	*KRAS* (90%); *MAP2K4*	Almoguera *et al*[54]; Hruban *et al*[56]; Pellegata *et al*[57]; Hezel *et al*[58]; Maitra *et al*[59]
DNA damage repair	*TP53* (75%-90%)	Almoguera *et al*[54]; Redston *et al*[67]; Olive *et al*[68]
TGF-β	*DPC4* (50%), *SMAD4*	Almoguera *et al*[54]; Yachida *et al*[73]
Apoptosis	*CASP10*; *CAD*	Jones *et al*[77]
Cell adhesion	*FAT*; *PCDH9*	Jones *et al*[77]
Hedgehog	*GLI1*; *GLI3*	Jones *et al*[77]
Integrin	*ILK*; *LAMA1*	Jones *et al*[77]
JNK	*MAP4K3*; *TNF*	Jones *et al*[77]
Small GTPases	*PLCB3*; *RP1*	Jones *et al*[77]
Wnt-β-catenin	*MYC*; *TSC2*	Jones *et al*[77]

APC: Adenomatous polyposis coli; CDK2NA: Cyclin-dependent kinase inhibitor 2 A; CAD: Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase; CASP10: Caspase 10; DPC4: deleted in pancreatic cancer, locus 4; FAT: Fat tumor suppressor; GLI 1: Glioma-associated oncogene; GLI3: Glioma-associated oncogene 3; GTPases: Guanosine triphosphate; Wnt-B-catenin: Wingless type B-catenin; ILK: Integrin-linked kinase; JNK: c-Jun N-ternmial kinases; KRAS: Kristen rat sarcoma; LAMA1: Laminin A-1 chain; MAP2K4: Mitogen-activated protein kinase kinase 4; MAP4K3: Mitogen-activated protein-3 kinase-3: TNF: Tumor necrosis factor; MYC: Myelocytomatosis oncogene; PCDH9: Procadherin 9; PLCB3: Phospholipase C, beta 3; RP1: Retinitis pigmentosa 1; SMAD4: Mothers against decapentaplegic homolog 4; TGF-β: Transforming growth factor β; TP53: Tumor protein 53; TSC2: Tuberous sclerosis 2; RAS: Rat sarcoma.

that has shown a great deal of promise in therapeutic monitoring and in identifying the recurrence of pancreatic cancer after treatment is the carbohydrate antigen 19-9 (CA 19-9), a sialylated Lewis blood group A antigen secreted by many pancreatic lesions[37]. Yet, CA 19-9 is not specific for pancreatic cancer and therefore cannot be used to screen for this tumor. Several other conditions, including hepatobiliary diseases, pancreatic diseases and gastrointestinal malignancies, bronchitis, congestive heart failure, cystic fibrosis, diverticulitis, lung cancer, ovarian cysts, pleural effusions, renal cysts and rheumatoid arthritis may present with elevated levels of CA 19-9[38]. In addition, approximately 10% of patients with pancreatic cancer are negative for Lewis antigen a or b. As a result, these patients are unable to synthesize CA 19-9 and will have no detectable levels of this biomarker, even in advanced stages of pancreatic cancer[39]. Although measurement of serum CA 19-9 levels has clinical significance in determining treatment and prognosis for patients with known pancreatic cancer, its usefulness as a diagnostic screening tool of the disease is not substantiated[40].

Upon diagnosis of pancreatic cancer, treatment and management of patients should utilize a multidisciplinary team including, primary care physicians, medical oncolo-

gists, radiation oncologists, surgeons, endocrinologists, radiologists and pathologists[41]. Cancer staging subsequently follows, with the American Joint Committee on Cancer providing the standard model, based upon the tumor-node-metastasis system[42]. However, not all criteria regarding tumor staging can be measured prior to surgical intervention. As a result, the majority of staging for pancreatic cancer incorporates imaging results and liver function tests. From these results, patients with pancreatic cancer can often be classified into three major cohorts: (1) patients with a resectable tumor or borderline resectable tumor; (2) patients with a locally invasive tumor without metastasis; and (3) patients with a systemically disseminated tumor.

Appropriately designating cases into the proper subgroup is vital to ensure appropriate treatment and management of patients presenting with pancreatic cancer. Often, fine needle aspiration guided by endoscopic ultrasonography is necessary to obtain histological confirmation[43,44], especially prior to the initiation of chemotherapy and radiation. Throughout the treatment process, CA 19-9 should be continuously measured[45,46]. Nonetheless, previous studies still support the usefulness of CA 19-9 in predicting patient response to chemotherapy[47,48], preoperative prognosis[49], as well as assessing treatment response[50,51], overall survival[51-53], and recurrence[51].

THERAPEUTIC RESISTANCE IN PANCREATIC CANCER

Cellular mechanisms of therapeutic resistance

In an effort to understand the therapeutic resistant (Table 1) nature of PDA, researchers have attempted to characterize the molecular and cellular components of the pancreatic cancer cells as well as the desmoplasia that surrounds them. Although pancreatic cancer displays pathologic and clinical heterogeneity, data suggests the majority of PDA express a successive accumulation of highly penetrant genetic alterations that occur at four genetic loci: K-ras, p53, cdkn2a and smad4/DPC4[54]. Originating in the ductal epithelium, pancreatic cancer evolves from a premalignant lesion to a highly invasive metastatic disease[55].

Ninety percent of tumors have point mutations that are specific for the KRAS2 oncogene, resulting in the constitutive production of the Ras protein[56-59]. Occurring early in tumorogenesis, these point mutations are essential for maintaining the malignant phenotype because once activated, Ras initiates a signal transduction cascade that activates proliferative and cell survival pathways and increases cell invasion[60,61]. The majority of the point mutations occur on codon 12 of the ras protein and give rise to pancreatic tumor-specific neo-antigens. Several studies demonstrated these antigens are recognized by helper T-cells and cytotoxic T-cells[62,63]. Using this knowledge, scientists developed personalized peptide vaccines corresponding to the K-ras mutations present in the tumors of patients enrolled in one clinical trial[64]. The vaccine was

Table 2 Extracellular mechanisms of thereapeutic resistance in pancreatic cancer

Potential therapeutic targets	Extracellular response	Ref.
K-ras mutant oncogene	Proliferation of desmoplastic reaction (leukocytes, fibroblasts, endothelial cells, neuronal cells, collagen, hyaluron): upregulation of GM-CSF	Chu *et al*[78]; Neesse *et al*[79]; Ying *et al*[81]; Nolan-Stevaux *et al*[82]; Bayne *et al*[87]
Sonic hedgehog (SHH)	Growth and differenatiation of stromal fibroblasts	Bailey *et al*[83]; Tian *et al*[84]; Olive *et al*[85]
Tumor associated macrophages (TAMs); cancer associated fibroblasts (CAFs); regulatory T-cells (Treg); myeloid derived suppressor cells	Evasion of the immune system	Bayne *et al*[87]; Pylayeva-Gupta *et al*[88]
Desmoplastic reaction	Anti-angiogenesis; hypoxic tumor environment	Komar *et al*[86]

K-ras: Kinase-rat sarcoma; GM-CSF: Granulocyte macrophage colon-stimulating factor.

proven safe and tolerable and resulted in a more efficient immunologic attack on the tumor[65]. As a result, patients given the vaccine demonstrated a more favorable clinical outcome than those not given the vaccine. Combined with surgical resection, a long-term immune response initiated by the K-ras vaccine has resulted in a 10-year survival in some patients. These results may implicate a role for the K-ras vaccine as an adjuvant treatment option in the future[66,67].

Rather than being activated like the mutated KRAS2 oncogene, the p53 tumor suppressor gene is inactivated in 75%-90% of pancreatic tumors[68,69]. As a result, there is an impaired response to DNA damage in pancreatic epithelial cells, impaired apoptosis and impaired cell cycle control[70,71]. Two other tumor suppressor genes, *p16Ink4a* and *p15ARF* are encoded by the cdkn2a locus. Inactivation mutations in these genes are present in about 90% of human PDA[72,73].

A fourth common mutation seen in more than half of pancreatic cancers causes an alteration in DPC4[74]. These mutations confer a metastatic phenotype. The genetic makeup of the patient determines the number and combination of these mutations that will be present in their PDA. Patients with three or four mutated genes will have a much worse prognosis than those with one or two. The variable expressivity of these tumors presents a challenge in effectively treating them[75].

In addition to these four primary genetic alterations in PDA many other less-frequent mutations occur as well[76-78]. According to a comprehensive genetic analysis of 24 pancreatic cancers, an average of more than 60 genetic abnormalities, primarily point mutations, per tumor were noted in the PDA phenotype. While these mutations have been organized into 12 functional cancer-relevant pathways, not all tumors have alterations in each of these pathways. In addition, key mutations in select pathways appear to differ from one tumor to another[78]. These pathways may confer therapeutic resistance in the pancreatic tumor. Significant genomic instability in pancreatic cancer may also reduce the effectiveness of therapeutic agents by contributing to acquired chemoresistance.

Extracellular mechanisms of therapeutic resistance

Paracrine signals from pancreatic cancer cells stimulate the extracellular proliferation of leukocytes, fibroblasts, endothelial cells, neuronal cells, collagen and hyaluron (Table 2). This extracellular proliferation of cells is known as a desmoplastic reaction. It forms a thick stromal environment around the pancreatic cancer cells[79,80]. Studies have demonstrated that the signals that influence the proliferation of the desmoplastic reaction originate from the K-ras mutant oncogene in the epithelium of the pancreatic cancer cells[81,82].

In addition to the K-ras mutant signaling pathway, there has been an effort by researchers to understand the roles of other signaling pathways between the pancreatic cancer cells and their microenvironment. Sonic hedgehog (SHH) functions similarly to the K-ras mutant. Although it is over expressed in pancreatic cancer cells during the early stages of their development, SHH does not act on the SHH pathway in these cells[83]. Instead, it acts in a paracrine fashion in the extracellular fibroblasts, resulting in their growth and differentiation[84,85].

The desmoplastic reaction not only provides a mechanical barrier to the pancreatic cancer cells, but it is also thought to contribute to the anti-angiogenic environment that is characteristic of pancreatic ductal adenocarcinoma. Both properties directly affect therapeutic efficacy. Inadequate drug delivery to the site of the tumor is directly correlated with a negative patient outcome[86].

Researchers have also suggested a role for the tumor stroma in the T-lymphocyte depleted microenvironment of the PDA. Several cell types found in the desmoplastic reaction have been associated with tumor associated macrophages, cancer associated fibroblasts, regulatory T-cells and myeloid derived suppressor cells. In addition, a role for a K-ras dependent signaling molecule has been shown to up-regulate granulocyte-macrophage colony stimulating factor when activated. This cytokine promotes the maturation of immature myeloid progenitor cells into myeloid derived suppressor cells[87,88].

TREATMENT OF PANCREATIC CANCER

Surgical resection

Although surgical resection offers hope for curative therapy, only 20% of patients present with potentially resectable tumors[89,90]. It is important to note that surgical resection is only considered in patients with completely

Table 3 Therapies for the management of pancreatic cancer

Therapeutic option	Subset	Ref.
Surgical resection	Cephalic pancreatoduodenectomy	Hidalgo[41]
	Distal pancreatectomy	
	Total pancreatectomy	
Chemotherapy	Neoadjuvant	Lemmens *et al*[101];
	Gemcitabine	Gillen *et al*[102];
	Adjuvant	Neoptolemos *et al*[108];
	Gemcitabine	Burris *et al*[118];
	5-Fluorouracil	Heinemann *et al*[119];
	Advanced disease	Reni *et al*[120];
	Gemcitabine	Moore *et al*[122];
	Gemcitabine + fluropyrimidines	Neesse *et al*[79]
	Gemcitabine + platinum analogs	
	Gemcitabine + erlotinib	
	FOLFIRINOX	
	Nab-paclitaxel	
Radiation therapy	Neoadjuvant	Pisters *et al*[131];
	Radiation + 5-fluorouracil	Hong *et al*[133];
	Radiation + paclitaxel	Yeo *et al*[140];
	Proton beam radiation + capecitabine	Regine *et al*[138];
	Adjuvant	Neoptolemos *et al*[137];
	Radiation + 5-Fluorouracil	Moertel *et al*[144];
	Radiation + gemcitabine	Schellenberg *et al*[147]
	Radiation + chemotherapy	
	Advanced	
	Radiation + 5-fluorouracil	
	Radiation + chemotherapy	
	Stereotactic body radiotherapy	
Personalized therapy	Target specific point mutations	Jones *et al*[77];
	Mitomycin C	Villarroel *et al*[153];
	Immune system stimulation	Yanagimoto *et al*[154]

resectable or borderline-resectable tumors (Table 3). Depending on the size and location of the tumor, three operative procedures are potentially utilized, as noted by Hidalgo[41], with additional removal of adjacent lymph nodes: (1) cephalic pancreatoduodenectomy (whipple procedure); (2) distal pancreatectomy; and (3) total pancreatectomy.

Although additional palliative care is often utilized, controversy surrounds the potential benefits. For example, almost 80% of patients presenting with tumors in the pancreatic head exhibit jaundice due to biliary obstruction[91,92]. However, previous investigations have conflicting results regarding preoperative biliary drainage with certain studies reporting a decrease in perioperative morbidity and mortality[93] while others concluding recognizable benefit[94,95].

Preoperative biliary stenting doubled between 1992 and 2007 due to evidence demonstrating a higher risk of postoperative complications in patients presenting with a tumor in the head of the pancreas. Biliary drainage was further supported by evidence demonstrating its ability to improve liver function, nutritional status and cell-mediated immune function[93]. Despite intentions to reduce post-operative morbidity and mortality by improving liver function, extensive clinical studies have demonstrated preoperative biliary stenting prolongs time to operation, increases preoperative infection and is associated with overall increased complication rates after surgical proce-

dures[94,95]. As a result, most studies have advised against routinely performing preoperative biliary drainage and have recommended that patients presenting with jaundice due to a resectable and non-metastatic tumor in the head of the pancreas should undergo early surgery without preoperative biliary resection[95]. Currently, the only indications for preoperative biliary decompression are for patients who present with severe jaundice, are undergoing neoadjuvant therapy, or have had their surgery postponed due to logistics[94,95].

Several poor predictors for successful resection have been identified, including lymph node involvement[96], high tumor grade[97], large tumor size[98], elevated CA 19-9 levels[46], and positive margins of tumor following resection[41]. These same factors also contribute to recurrence of pancreatic tumors. Even with surgical resection, 5-year survival rates remain dismal, at approximately 20% following surgery[90]. However, perioperative complications and mortality have significantly decreased over the past decade, likely due to greater hospital clinical volume through centralization[99,100].

Chemotherapy
Neoadjuvant therapy: Certain patients might receive neoadjuvant therapy, especially if the tumor presents with borderline resectability. In a study utilizing gemcitabine-based chemotherapy, improved tumor resection with increased survival rates was noted in border-line resect-

able cases[101,102]. However, these effects may only occur in select tumors, with influences by both the genetic composition and microenvironment of the pancreatic cancer[103,104]. For example, White *et al*[105] noted p53 mutations were more common in patients with large residual tumors following treatment with chemoradiation. Moreover, outcomes for neoadjuvant therapy prior to surgically-resectable tumors did not differ when chemotherapy was provided post-operatively[106]. Chemotherapy with radiation has also been shown to improve survival, but not stage, of cases presenting with locally invasive tumors without metastasis[107]. However, previous studies do note that surgical interventions are more challenging and increased postoperative stay is associated with patients undergoing resection after neoadjuvant chemoradiation therapy for locally invasive cancer[106]. Since metastatic pancreatic cancer cannot be completely resected, surgical options are unavailable and hence no neoadjuvant therapy can be provided. Lastly, it is important to note that prior to initiating neoadjuvant therapy, histological confirmation of pancreatic adenocarcinoma is required, unlike surgical resection, which often relies solely on imaging.

Adjuvant chemotherapy: Even following complete, successful resection of pancreatic tumors, overall survival and prognosis remains discouraging. Hence, postoperative chemotherapy or chemoradiation is almost always incorporated in the therapeutic regimen. Postoperative chemotherapy often utilizes gemcitabine or 5-fluorouracil (with concurrent leucovorin as a rescue agent). Both drugs have demonstrated significant increases in patient survival, regardless of initial case presentation. These drugs may also be given simultaneously, however, significant toxicity (especially gastrointestinal) has been reported. Although gemcitabine has often been considered the standard, previous studies do differ on which agents are associated with the most optimal benefits. In a phase III, randomized control trial, Neoptolemos *et al*[108] noted no significance difference in survivorship between gemcitabine and 5-fluorouracil (with folinic acid) in patients with resected tumors. In a separate study published in JAMA, the authors concluded that gemcitabine alone should be favored over 5-fluorouracil with leucovorin due to its decreased toxicity[108].

Developments of other complementary agents to enhance chemotherapeutic effects are currently under review. For example, possible inhibition of Hedgehog signaling[84] or concurrent use of Smac mimetics[109], microRNAs[110], Resveratrol[111], capecitabine[112], thymoquinone[113] or heat-shock protein complements[114] may promote tumor uptake and damage of administered drugs, such as Gemcitabine administered with concurrent curcumin may also be a potential option, especially in tumors exhibiting gemcitabine-resistance[115]. In addition to utilizing CA 19-9 and imaging to monitor patient response to chemotherapy, other markers, such as human equilibrative nucleoside transporter 1 levels have also shown to be useful[116]. Other gene expression levels, as noted in Fujita

et al[117], may also be predictive of treatment efficacy, particularly with gemcitabine. Further investigation is required as to whether adjuvant chemotherapy should be administered if prior neoadjuvant therapy before surgery had already been provided.

Chemotherapy for advanced disease: Due to its poor detection rate, 60%-70% of patients present with metastatic pancreatic cancer upon initial diagnosis. In the advanced stage of disease, pancreatic cancer causes imminent mortality for the majority of affected patients and median survival rate is typically 6-8 mo. Therefore, treatment of patients with metastatic pancreatic adenocarcinoma incorporates chemotherapy, targeted-therapy, comorbid conditions, intensive supportive treatment and psychosocial support.

Gemcitabine is currently considered the chemotherapeutic standard of care in treatment of advanced pancreatic cancer[118]. It has been shown to prolong the average survival rate by 4 mo. In an attempt to improve survival rates, several phase II and phase III trials combined Gemcitabine with fluoropyrimidines and platinum analogs. Most of these combinations failed to show statistically significant survival benefit, however compared to Gemcitabine alone[119]. In another attempt to prolong patient survival, scientists have developed several Gemcitabine-based polychemotherapy regimens involving 3-4 cytotoxic agents. When Reni *et al*[120] performed a randomized trial to test the cisplatin, epirubicin, fluorouracil and gemcitabine (PEFG-regimen) against gemcitabine alone, those patients treated with the PEFG saw a significant decrease in cancer progression, when compared to those treated with gemcitabine alone[120]. Yet in regards to survival, the infusional 5-FU/folinic acid, irinotecan, and oxaliplatin (FOLFIRINOX) regimen has been shown to be superior to the PEFG-regimen.

According to Conroy *et al*[121], FOLFIRINOX is the new standard in the treatment of advanced stage pancreatic cancer. Compared to gemcitabine alone, FOLFIRINOX demonstrated a better objective response rate, progression-free survival, overall survival and one-year survival. While the toxicity levels associated with FOLFIRINOX are greater than those caused by gemcitabine, the effects did not seem to have a significant impact on quality of life. In addition, few toxic deaths have been reported.

Inhibitors of epidermal growth factor receptor (EGFR) have also been tested for treatment of metastatic pancreatic cancer. Cetuximab, an anti-EGFR directed antibody and erlotinib, an oral EGFR tyrosine kinase inhibitor have been tested in several randomized trials. However, Moore *et al*[122] demonstrated that combining gemcitabine with erlotinib is the only targeted-therapeutic agent that has clinical efficacy. Compared with gemcitabine alone, gemcitabine plus erlotinib showed significant decrease in tumor progression and concurrently increased overall survival rates.

Other targeted therapies have focused on targeting

the desmoplastic stroma, one of the key components of pancreatic cancer that may contribute to impaired drug delivery and thus chemotherapy resistance[79]. Nab-pacli-taxel is one therapy that has been developed to diminish this stromal tissue network. Studies have demonstrated that albumin interacts with secreted protein acidic and rich in cysteine (SPARC), a matrix glycoprotein that has a role in tumor invasion, facilitating the uptake of pacli-taxel by the tumor[123]. Infante *et al*[124] have demonstrated that overexpression of SPARC in peritumoral fibroblasts was a negative prognostic indicator in patients with ad-vanced pancreatic cancer[124]. In a phase Ⅰ/Ⅱ trial, Von Hoff *et al*[125] demonstrated the ability of nab-paclitaxel to increase median survival rate in patients with metastatic pancreatic cancer.

Similar to the mechanism of action of nab-paclitaxel, new therapies are being developed that target the peritu-moral stroma in order to increase tumor perfusion. One such preclinical strategy inhibits the hedgehog signaling pathway, depleting the stroma and increasing angiogen-esis to improve delivery of chemotherapeutic agents to the tumor[126].

Phase Ⅱ clinical trials have demonstrated a benefit of second-line treatment for patients who are resistant to gemcitabine treatment[127]. Second-line treatments typi-cally consist of fluoropyrimidines in combination with oxaliplatin[128]. Limited data exists on how to treat patients who do not tolerate FOLFIRINOX as a first-line therapy. However, Conroy *et al*[121] have demonstrated the benefit of using gemcitabine-based therapies in these instances.

The primary prognostic indicators for patient survival are both patient and tumor related. Based on the genetic and morphologic heterogeneity that exists within each individual pancreatic tumor, therapy, dose and length of therapy administration will need to be customized for each Individual patient to ensure optimal treatment.

Radiation

Neoadjuvant radiation therapy: Many studies have demonstrated the important roles for chemotherapy and radiation therapy in preventing the recurrence and improving the resectability of pancreatic tumors. While surgery is currently the only potential curative treat-ment modality for pancreatic cancer, more than 80% of patients who undergo surgical resection will experience tumor recurrence within 12 mo of their procedure[129]. Therefore, a great deal of focus has not only been placed on developing effective neoadjuvant and adjuvant thera-pies, but also on effective preoperative staging techniques to determine candidates who will benefit most from sur-gical resection[130]. Since surgery is associated with high rates of morbidity and mortality, many patients do not begin adjuvant therapy until after they have recovered. As a result, there is a long delay before they receive adjuvant therapy. In order to begin more potent treatments earlier and to potentially shrink the tumor before surgery, re-searchers developed neoadjuvant therapeutic regimens.

Multiple trials of 5-fluorouracil-based chemoradia-

tion have been done to date. At the conclusion of these studies, researchers determined that a combined treat-ment modality with preoperative rapid-fractionation chemoradiation, Whipple procedure, and intra-operative radiation therapy resulted in minimal toxicity and a small recurrence rate[131]. In a follow-up study, paclitaxel re-placed 5-fluorouracil and was used to treat 35 patients who presented with resectable pancreatic tumors[132]. Based on the results of this study, researchers concluded that preoperative paclitaxel-based chemotherapy with rapid-fractionation chemoradiation, Whipple procedure and intraoperative radiation therapy resulted in similar outcomes as the previous study, but toxicity levels were greater than those from 5-fluorouracil. In another study, researchers treated patients who presented with tumors in the head of the pancreas with a neoadjuvant chemora-diation regimen of capecitabine with proton beam radia-tion[133]. No dose limiting toxicities were observed and the authors concluded that this form of neoadjuvant therapy was feasible. In several other prospective neoadjuvant chemoradiation trials in patients with resectable pancre-atic cancers, the rate of resection was high in all studies, ranging from 87%-100%[107,134,135].

Adjuvant radiation therapy: The median survival rate of patients who undergo surgical resection of a pancre-atic tumor is 15-22 mo. Only 20% of patients survive for five years following surgery[136]. The most common site for pancreatic cancer recurrence is the retroperitoneum. Therefore, adjuvant therapy is needed to improve patient prognosis. In the United States, adjuvant therapy is cur-rently delivered in the form of chemotherapy, chemoradio-therapy or chemotherapy followed by chemoradiotherapy. Standard adjuvant treatment in Europe is chemotherapy alone. These guidelines were based on previous random-ized trials that showed improved survival in patients given adjuvant therapy following surgical resection.

The first prospective trial for adjuvant chemoradio-therapy was conducted by the Gastrointestinal Tumor Study Group in 1985. The trial enrolled patients with resectable pancreatic cancer. The protocol called for external beam radiation delivered with 5-fluorouracil. The patients were then given a maintenance dose of 5-fluoruracil for an additional two years following initial treatment. Patients treated with adjuvant chemoradiation achieved a longer median and 2-year survival rate than those not treated with adjuvant therapy. As a result, ad-juvant chemoradiation became the most frequently used adjuvant treatment for resectable pancreatic cancer in the United States.

To further assess adjuvant radiation therapy for re-sectable pancreatic cancer, the European Study Group for Pancreatic Cancer (ESPAC-1) conducted the largest randomized trial to date in 2004[137]. In order to evalu-ate the effects of chemoradiotherapy and chemotherapy on patient survival following surgical resection, patients with resectable pancreatic ductal adenocarcinoma were divided into one of four groups: chemotherapy alone;

chemoradiotherapy alone; chemoradiotherapy followed by chemotherapy; or no further treatment. Patients who received chemotherapy followed by chemoradiotherapy had a 5-year survival rate that was 10% less than those who received chemotherapy alone. In addition, patients who received chemotherapy treatment showed a 5-year survival benefit when compared to those who received no chemotherapy. As a result of these findings, the standard of adjuvant treatment in Europe shifted towards chemotherapy only, abandoning postoperative chemoradiation.

A phase Ⅲ trial was conducted by the Radiation Oncology Group and GI Intergroup around the same time as the ESPAC-1 trial[138]. This trial compared the 5-fluorouracil-based chemoradiation to gemcitabine-based chemoradiation. Patients receiving gemicitabine-based chemoradiation had a median survival of 20.6 mo, 3.5 mo more than those given 5-flurouracil-based chemotherapy. The Charite Onkologie Clinical Studies in GI Cancer 001 (CONKO-001) trial in Germany and Austria showed similar median survival in patients given gemcitabine-based chemotherapy alone[139].

Additionally, reports from several institutions, including the Mayo Clinic, Johns Hopkins Medical Center and Virginia Mason University have all reported the benefit of adjuvant chemoradiation therapy following resectable pancreatic cancer compared to those who received surgery alone[140-142].

Management of locally advanced pancreatic cancer: Patients with locally advanced pancreatic cancer achieve little benefit from surgical resection because their cancer meets the criteria for unresectable cancer: (1) distant metastasis and/or pancreatic lymph node involvement; (2) encasement or occlusion of the superior mesenteric vein or superior mesenteric vein/portal vein confluence; and/or (3) direct involvement of the celiac axis, aorta, inferior vena cava, or superior mesenteric artery[143]. As a result, chemoradiation is recommended for these patients based on data from several studies.

A 1981 trial conducted by the Gastrointestinal Tumor Study Group compared the effects of high-dose radiation therapy alone; moderate dose radiation combined with 5-flurorouracil and high dose radiation combined with 5-fluorouracil in 194 patients with locally advanced pancreatic cancer. Researchers found that patients administered 5-fluorouracil in combination with low or high dose radiation showed a greater median survival than those treated with radiation alone[144]. In a follow-up study, the same group demonstrated that chemotherapy, when combined with radiotherapy afforded patients with locally advanced pancreatic cancer a greater median survival when compared to combination chemotherapy alone[145]. These results were verified by the ECOG trial as well, which demonstrated an increased median survival rate in patients treated with gemcitabine and radiotherapy together as opposed to those treated with gemcitabine alone[146].

Although chemoradiation has been shown to provide an increased median survival in patients with locally advanced pancreatic cancer by 9-13 mo, many of these patients progress to the metastatic stage of disease shortly after therapy. Perhaps a better approach to these patients would be to begin a chemotherapy regimen, restage their cancer after completion of initial treatment, and follow up with chemoradiation in patients who do not demonstrate metastatic disease progression. Radiation in these patients could relieve pain associated with disease by slowing local progression.

Stereotactic body radiotherapy: An evolving radiation therapy for treatment of locally advanced pancreatic cancer is stereotactic body radiation therapy. This newer technique uses image guidance to deliver toxic radiation doses directly to tumors. As a result, there is less systemic involvement, and patient outcomes are improved without having to undergo daily treatments. However, the major challenge of this novel therapy is accurately characterizing the tumor in terms of size, number and location. In order to do so, precise diagnostic tests and real-time imaging techniques are used. In addition, each treatment regimen is tailored to each individual patient. To date, scientific literature suggests sterotactic body radiotherapy does slow local progression in patients with locally advanced pancreatic cancer[147-149]. However, it does not increase overall survival rate because patient mortality is due to distant metastases.

Advances in radiation therapy techniques: Over the past decade, major advances in radiation therapy have been in treatment planning and more precise delivery methodologies. One technique, intensity-modulated radiotherapy, decreases systemic toxicities in patients by modifying radiation dose delivery specifically to the tumor sites, sparing surrounding normal tissue[150,151]. Another technique, image-guided readiotherapy, has provided more accurate visualization and real-time tracking of viscerally-located tumors and thus has enabled more precise delivery of high-dose therapeutic beams of radiation to these tumors and prevented adverse effects in normal tissue[152].

In order to improve patient outcome and prolong median survival rate, additional studies are needed to define the optimal role of adjuvant and neoadjuvant treatment in patients with resectable pancreatic cancer. As radiation therapies become more precise and customized to individual patients, it will be necessary to continue to investigate their future role in the treatment of pancreatic adenocarcinoma, especially as a greater understanding of the molecular pathways involved in the carcinogenesis and progression of this disease are understood.

Personalized therapy: In an article published in *Science*, Jones *et al*[77] performed a comprehensive genome assessment on 24 different pancreatic cancers. Results revealed an average of 63 genetic mutations per cancer, spanning 12 separate signal transduction pathways. This study sup-

ports the notion of pancreatic cancer being a genetically heterogeneous malignancy, partially accounting for its notable resistance to therapy as well as varied responses to treatment. Moreover, this finding likely explains why no candidate gene has been identified in the treatment of pancreatic cancer. This heterogeneity will likely dictate an individualized, unique approach for each particular case, which has already shown to be effective against even advanced pancreatic cancer stages. In one such case report, Villarroel *et al*[153] identified Mitomycin C, a DNA-damaging agent, as a highly effective agent by utilizing a xenograft derived from the patient's tumor. Upon administration of this drug, the patient exhibited notable clinical benefits for over three years, despite the tumor previously being gemcitabine resistant. Personalized immune system stimulation may also be a viable option in treatment of unresectable disease. For example, Yanagimoto *et al*[154] incorporated a vaccine containing individualized, reactive peptides with concurrent gemcitabine treatment, noting a significant correlation between immune boosting and survivorship.

Due to the ongoing advances in DNA sequencing, personalized genomic therapy appears more plausible. Moreover, as scientists continue to identify regions of the genome with high potential for tumor-pathogenesis, this method will only become more efficient. Upon identification, cases can be distributed into cohorts based upon their tumor's genetic composition and administered treatment previously demonstrated to be effective in that particular subgroup. This method would not only identify pertinent biomolecules in pancreatic pathogenesis, but also lead to tumor-specific treatment, which is likely necessary if we are to see any significant improvement in the prognosis of pancreatic cancer.

FUTURE PERSPECTIVES

Despite decades of effort by the scientific community to design sophisticated chemotherapeutic and radiation techniques to combat pancreatic ductal adenocarcinoma, less than 5% of patients with this disease have a 5-year survival rate. The majority of patients have a median survival period of 4-6 mo[155,156]. A combination of factors including few early symptoms, few accurate biomarkers for early detection, rapid metastasis to the lymphatic system and distant organs, and few effective treatment options, makes this disease one of the most deadly cancers today[157,158]. Although current therapeutic agents have had limited effects on patient care, there has been substantial advancement in the understanding of the molecular and biological makeup of pancreatic adenocarcinoma. This knowledge has the potential to lead to the development of novel therapies that could significantly improve the lifespan of individuals suffering with this disease.

Such advances in understanding this complex disease have been achieved with genetically-engineered mouse models and patient-derived xenografts. These studies have demonstrated the genetic diversity of pancreatic

ductal adenocarcinoma results from successive accumulation of mutations in several primary oncogenes and tumor suppressor genes, leading to its heterogeneity, instability and early tumor metastasis[77]. Pancreatic ductal adenocarcinoma is composed of several compartments. In addition to a mature cancer cell population, some researchers have characterized cancer cells that display stem cell properties and are resistant to chemotherapy and radiation therapy, potentiating their ability to metastasize[56]. Another area of interest is the dense tumor microenvironment that surrounds the pancreatic cancer cells. Composed of collagen I, activated fibroblasts, and inflammatory cells, it has been shown to interact with pancreatic cancer cells in order to foster tumor development, act as a barrier to optimal drug delivery and aid the tumor in invasion and metastasis[59]. Furthermore, this dense stroma creates a hypoxic microenvironment that pancreatic cancer cells thrive in[159]. However, the mechanisms by which these cancer cells adapt to these conditions are currently being identified and may serve as additional therapeutic targets in the near future.

Pancreatic cancer cells

Pancreatic ductal adenocarcinoma most likely originates in the ductal epithelium of pancreatic cells[160]. Neoplastic cells contain one or more of four primary genetic mutations that will ultimately give rise to the invasive form of this disease. Ninety percent of these tumors have mutations in the KRAS2 oncogene, resulting in the activation of proliferative survival signaling pathways. Ninety-five percent have a mutation in the CDKN2A tumor suppressor gene, resulting in the loss of the p16 protein and thus loss of regulation of the G_1-S transition of the cell cycle. An abnormal *TP53* gene has been identified in 50%-75% of characterized cancer cells, allowing cells to avoid DNA damage control checkpoints and subsequently, apoptotic signals. Another 50% have a deleted SMAD4/MADH4 gene, resulting in aberrant signaling by the TGF-β cell surface receptor[153].

One study performed genetic analysis on 24 pancreatic ductal adneocarcinomas and reported that each tumor has an average of 63 clinically relevant genetic abnormalities. While these abnormalities differ from one cancer to another, they all seem to play a role in 12 functional cancer-related pathways[161]. Recently, two studies compared the genetic makeup of distant metastases to their primary metastatic lesions. They found that over time, the distant metastases accumulated additional mutations to those present in the clonal cells from which they arose, adding to the complexity this disease[162]. Such genetic diversity not only results in different prognoses for patients, but also causes individual tumors to respond differently to common therapeutic agents used in treating pancreatic ductal adenocarcinoma[163].

The varying degrees of genetic instability that exist between individual pancreatic ductal adenocarcinomas present a greater need for genomic sequencing of individual tumors, followed by personalized therapies to target spe-

cific genes and pathways that have been altered[164]. Several clinical trials have begun exploring this idea[165]. In order to incorporate this treatment modality into the clinical setting, several criteria must be met: (1) a high quality tumor tissue sample must be attained at the time of diagnosis; (2) sophisticated bioinformatic analysis of the data must be performed to identify the most relevant mutations in each tissue sample; and (3) model systems must be designed to experimentally test varying treatment options to determine the most effective one for the patient. Perhaps the greatest challenge lies in developing a drug once specific genetic abnormalities have been identified.

Pancreatic cancer stem cells

Recently, investigators have characterized pancreatic cancer cells with stem cell properties[162]. Known as pancreatic cancer stem cells, these cells have the ability to regrow new tumors when placed into naïve mouse models and are able to maintain long-term tumorigenic potential[163]. Studies have shown that pancreatic CSCs are not only capable of self renewal, but may also confer therapeutic resistance, and play a role in tumor formation and disease progression[164,165]. In addition, different cancer stem cell populations perform different biological functions. One of the most recent findings has demonstrated that these cells may transition between epithelial and mesenchymal states, contributing to their highly metastitc potential[165]. Therefore, eliminating or inhibiting these CSCs with new therapeutic designs could significantly improve patient outcomes. Current therapies have already been designed to target cancer stem cell-specific antigens in order to inhibit their roles in cell survival, adhesion, self renewal and differentiation. A greater understanding of individual CSC populations and how they interact with one another will enable further progress in the treatment of pancreatic cancer[164,165].

Therapeutic targets of pancreatic ductal adenocarcinoma cancer stem cells include genes located in developmental pathways such as hedgehog, Wnt, Notch, CXCR4 and Met. In addition, targeting apoptotic pathways such as DR5 and nodal-activin could have a significant therapeutic implications. Several preclinical trials have been conducted to target these pathways in models of human pancreatic ductal adenocarcinoma cancer stem cells. By inhibiting these pathways, investigators were able to confer longer-term tumor control when compared to current standard chemotherapeutic regimens, in which tumor regression was significantly shorter-lived. In one recent trial, salinomycin was shown to induce cell death in epithelial-mesenchymal transition-induced cancer stem cells[124].

Due to the heterogeneity of the cancer stem cell population, future drugs designed to target pancreatic ductal adenocarcinoma stem cells may require clinical trials in which therapies are designed specifically for pancreatic tumors in each individual patient. These customized therapies could potentially serve as adjuvant treatment options for patients following pancreatic tumor resection. Similar to previous clinical trial designs, adjuvant thera-

pies targeting cancer stem cells could be given to patients with or without current conventional chemotherapy and/or chemoradiation to determine which option confers the greatest overall survival rate in patients following surgical resection.

Tumor microenvironment

One of the primary characteristics of pancreatic ductal adenocarcinoma is the dense stroma surrounding the pancreatic cancer cells. Composed of fibroblasts, collagen I and other fibrillar elements, this desmoplastic reaction has become a primary target of current drug therapies[125]. The key players in the formation and turnover of this dense stroma are pancreatic stellate cells. Certain growth factors [TGF-β1, platelet-derived growth factor (PDGF) and fibroblast growth factor] activate these cells to myofibroblasts. Not only do these activated myofibroblasts secrete components of the extracellular matrix, but they are also responsible for the poor vascularization of the pancreatic tumor[166,167]. In addition to forming a mechanical barrier around the pancreatic cancer cells, the stroma has an important role in tumor formation, progression, invasion and metastasis[168]. Many proteins expressed by stromal cells have been directly correlated with poor prognosis and resistance to current therapies [Cox-2, PDGF receptor, VEGF, stromal-derived factor, chemokines, integrins, secreted protein acidic and rich in cysteine (SPARC), and hedgehog elements].

Pre-clinical models have demonstrated that targeting these receptors and enzymes is associated with antitumor effects. Perhaps one of the most promising targets to date is the hedgehog signaling pathway. Some studies have demonstrated that targeting smoothened resulted in a depletion of the stroma and thereby increased delivery of gemcitabine to the tumor cells[169]. Another target for therapeutic trials has been SPARC (osteonectin) and hyaluronic acid. SPARC is an extracellular matrix protein that plays a role in collagen turnover in the dense stroma. It is associated with invasion and metastasis in pancreatic ductal adenocarcinoma, and thus poor prognosis in patients with elevated levels[170]. As mentioned previously, SPARC is the target of the albumin-bound chemotherapy agent, nab-paclitaxel. Phase I / II clinical trials have shown that administration of this drug breaks down the stroma and improves delivery of the chemotherapeutic agent to the site of the tumor[171]. In addition, in a mouse model of pancreatic ductal adenocarcinoma, investigators demonstrated that administration of pegylated hyaluronidase eliminated hyaluronic acid content, thereby relieving pressure on the blood vessels surrounding the tumor and allowed for increased perfusion of the chemotherapeutic agent to the site of the tumor[172].

The immunosuppressive nature of the tumor microenvironment has been another stromal characteristic targeted by recent therapeutic development. Using a CD40 antibody combined with gemcitabine chemotherapy, researchers have attempted to reverse immune suppression and drive antitumor T-cell responses in patients with non-resectable pancreatic ductal adenocarcinoma. Studies

have shown that this agent results in tumor regression by stimulating tumor macrophages to attack and deplete the pancreatic cancer stroma[173].

To date, targeting pancreatic ductal adenocarcinoma has proved most effective when treating patients with locally advanced disease, especially patients with tumors characterized by wild-type DPC4. These tumors are known to be less prone to metastasis and possess higher stromal content. Other tumors, especially those in late stages of the disease, characterized by distant metastases, have not been effectively treated with current stromal-targeting therapeutic agents. This is due to the fact that although pancreatic ductal adenocarcinoma has a rich and hypovascularized stroma, metastases arising from this cancer do not and are not different from other tumors. Therefore, patients who may benefit most from treatment with agents targeting the dense stroma microenvironment would be those with resectable tumors that have not progressed to the advanced stages of disease[174].

Metabolic pathways

Another conventional way to target pancreatic ductal adenocarcinoma would be to inhibit its major metabolic pathways. In order to do so, researchers would need to prevent its supply of glucose and glutamine; interrupt the pathways that enable it to exist in a hypoxic environment[175]; and prevent its ability to digest intracellular organelles for energy[176].

Investigators have identified several key metabolic enzymes to target [hexokinase, pyruvate kinase, lactate dehydrogenase A (LDHA) and ampicillin-activated protein kinase (AMPK)]. Several preclinical trials have demonstrated the anti-tumor effects of agents directed against these enzymes. One study demonstrated a potential clinical application for the LDHA inhibitor, FX11. By blocking the conversion of lactate to pyruvate in cells with p53 mutations, FX11 has antitumor potential. However, to date, there are only two therapies that have shown potential for targeting pancreatic ductal adenocarcinoma metabolism. One of these medications, metformin, is an activator of AMPK. It has been shown to decrease the potential for patients with diabetes to develop pancreatic cancer and to increase survival in diabetic patients with this disease[177,178]. The other drug used to target the metabolic pathways of is rapamycin. An inhibitor of mTOR, rapamycin has been shown to decrease glucose uptake by reducing levels of Glut1 in pancreatic cancer[179,180].

In order to inhibit autophagy, a significant mechanism for pancreatic cancer cell survival, investigators have used chloroquine, the antimalarial drug[181]. In preclinical trials with both allografts and xenografts, chloroquine has been shown to decrease tumorigenesis in a transgenic model and is currently being tested in clinical trials.

CONCLUSION

A greater understanding of the molecular and cellular makeup of pancreatic cancer over the past four decades has resulted in innovative therapeutic designs to target this aggressive malignancy. We now know that pancreatic cancer is a dynamic, heterogenous and genetically unstable tumor that results from successive mutations early in disease and gives rise to metastases that continue to garner mutations as they travel to distant locations. An equally important understanding of the role the peritumor microenvironment, composed of a dense desmoplastic stroma, plays in tumor development, metastases and as a barrier to chemotherapy delivery has been elucidated over the years. More recently, a role for pancreatic cancer stem cells in resistance to chemotherapy and radiation therapy was discovered. In addition, a deeper understanding of the metabolic pathways responsible for adaptation of pancreatic cancer cells to hypoxic environments has significant implications for future therapeutic development.

While some of these discoveries have resulted in novel therapeutic targets and treatment strategies and others are currently being tested in preclinical trials, efficient and effective drug development to combat pancreatic ductal adenocarcinoma is a necessity for the future. The majority of clinical trials have been conducted in patients with advanced stage disease. In the future, it will be necessary to design clinical trials to enroll patients with earlier stages of pancreatic cancer in an attempt to cure their cancer before it can metastasize.

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Complex role for the immune system in initiation and progression of pancreatic cancer

Kristin S Inman, Amanda A Francis, Nicole R Murray

Kristin S Inman, Amanda A Francis, Nicole R Murray, Department of Cancer Biology, Mayo Clinic, Jacksonville, FL 32224, United States
Author contributions: Inman KS performed the research; Inman KS and Francis AA analyzed the data; Inman KS and Murray NR wrote the review.
Supported by The National Institute of Health (NIH/NCI R01CA140290-3 to Murray NR)
Correspondence to: Nicole R Murray, PhD, Consultant, Associate Professor, Department of Cancer Biology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States. murray.nicole@mayo.edu
Telephone: +1-904-9536108 Fax: +1-904-9536233

Abstract

The immune system plays a complex role in the development and progression of pancreatic cancer. Inflammation can promote the formation of premalignant lesions and accelerate pancreatic cancer development. Conversely, pancreatic cancer is characterized by an immunosuppressive environment, which is thought to promote tumor progression and invasion. Here we review the current literature describing the role of the immune response in the progressive development of pancreatic cancer, with a focus on the mechanisms that drive recruitment and activation of immune cells at the tumor site, and our current understanding of the function of the immune cell types at the tumor. Recent clinical and preclinical data are reviewed, detailing the involvement of the immune response in pancreatitis and pancreatic cancer, including the role of specific cytokines and implications for disease outcome. Acute pancreatitis is characterized by a predominantly innate immune response, while chronic pancreatitis elicits an immune response that involves both innate and adaptive immune cells, and often results in profound systemic immune-suppression. Pancreatic adenocarcinoma is characterized by marked immune dysfunction driven by immunosuppressive cell types, tumor-promoting immune cells, and defective or absent inflammatory cells. Recent studies reveal that immune cells interact with cancer stem cells and tumor stromal cells, and these interactions have an impact on development and progression of pancreatic ductal adenocarcinoma (PDAC). Finally, current PDAC therapies are reviewed and the potential for harnessing the actions of the immune response to assist in targeting pancreatic cancer using immunotherapy is discussed.

Core tip: The development and progression of pancreatic cancer is heavily influenced by the immune response. Inflammation of the pancreas (pancreatitis) is a significant risk factor for pancreatic cancer. Immune cells recruited to the inflamed pancreas release additional cytokines and potentiate damage to the tissue. Pancreatic cancer is characterized by profound immune suppression thought to be caused by signals originating from the tumor cells. Additionally, a subset of immune cells has been shown to support the growth of pancreatic cancer cells. Novel therapies for pancreatic cancer aim to utilize this unique immune environment to target this deadly disease.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, and with a 5-year survival rate of 6%, it is one of the deadliest cancers worldwide[1]. The development and progression of PDAC is strongly influenced by the presence of inflammation[2]. Inflammation of the pancreas (pancreatitis) is a strong risk factor for PDAC development[3,4], and has been described as a critical factor in the initiation[5] and maintenance[6,7] of pancreatic disease. Additionally, PDAC is characterized by marked immunosuppression[6], which is thought to promote tumor progression and invasion.

This review highlights the role(s) of the immune response in the development of PDAC, focusing primarily on inflammation. Inflammatory conditions of the pancreas that can lead to increased risk for PDAC (pancreatitis), as well as the role of the immune response in the progressive stages of pancreas disease development, are discussed. Data from clinical studies and rodent models are integrated to present an up-to-date consensus of the role of inflammation in the initiation and progression of pancreatic cancer.

IMMUNE SYSTEM: A BRIEF OVERVIEW

The immune system is characteristically activated in response to infection by a foreign pathogen. In the case of cancer, tumor-specific antigens are recognized by the immune system, turning the cancer cell, in essence, into a foreign pathogen[8]. This allows the immune system to act as an extrinsic tumor-suppressor system. However, over time the chronic immune response to the tumor drives immunoselection of tumor cells that are able to thrive in an immunocompetent environment[9], much like the resistant bacterial strains that emerge as a result of exposure to antibiotics.

The mechanism by which the immune system can initially protect a host from tumor growth, but in some cases subsequently promotes cancer progression, is termed cancer immunoediting[10]. Cancer immunoediting is a dynamic process consisting of three phases, (1) elimination, when the immune system overcomes and eliminates the tumor before it can progress to a clinically relevant disease; (2) equilibrium, when the immune system does not eliminate the tumor, but controls tumor growth; and (3) escape, which occurs when the tumor has overcome the immune system and progresses to a clinically apparent disease. This third stage is generally seen as a failure in the adaptive immune system to provide long-term protection from tumor development due to selection of less immunogenic tumor cell variants during the equilibrium stage. Additionally, tumor escape can be facilitated by active immunosuppression induced by the tumor itself or some form of immune compromise or immune deficiency[11]. It is through this process that the cells of the immune system can act as both friend and foe to the body in the face of cancer.

Innate immunity

The innate immune system is composed of those immune cells that are already present in the body and can be immediately recruited to a site of infection during the process of "inflammation". Innate immune cells include granulocytes, macrophages, mast cells, natural killer cells (NKCs) and dendritic cells (DCs). Table 1 reviews the mechanism(s) of action attributed to the innate immune cells in both the normal immune response and during the process of cancer development. Neutrophils are by far the most abundant granulocytes in the body and typically one of the first cell types to respond; they secrete cytokines and chemokines, modulating other cells in the immune response[12]. Macrophages, another major cell type of the innate immune response, remove dead or dying cells and associated debris *via* phagocytosis, as well as play a role in the adaptive immune response. Macrophage maturation in response to various signals produces one of two cell types, M1-polarized cells, which initiate the inflammatory response, and M2-polarized cells, which restrain the inflammatory response[13]. M2-polarized macrophages are immunosuppressive and can limit adaptive immunity by inhibiting T-lymphocyte proliferation, thus impeding the T-lymphocyte response[14].

In the context of cancer, two types of macrophages emerge, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSC). Both cell types are similar to M2 macrophages and are recruited by tumor cells to thwart the anti-tumor immune activity. TAMs inhibit T-lymphocyte responses[15] and secrete cytokines that promote the tumor phenotype and metastasis[16,17]. In addition to their ability to directly induce T-lymphocyte apoptosis[18], TAMs produce arginase-1[19], a metalloenzyme that metabolizes and depletes the environment of arginine, an essential compound for T-lymphocyte proliferation[20,21].

The second type of immunosuppressive macrophage often found in the tumor microenvironment is the MDSC. Similar to TAMs in function, they differ mainly by their cell surface markers. MDSCs have been shown to exhibit powerful immunosuppressive properties, in part through production of reactive oxygen species and peroxynitrite[22,23], expression of arginase-1[24,25], induction of regulatory T-lymphocytes (Tregs)[26], and depletion of available cysteine, another amino acid required by T-lymphocytes[27].

NKCs are circulating immune cells that are able to kill target cells *via* induction of programmed cell death. NKCs have been shown to eliminate tumor cells, and treated cancer patients with high circulating NKCs have significantly longer metastasis-free survival[28]. The gap between the innate and adaptive immune systems is bridged by DCs[29]. Activated, antigen-presenting-DCs travel to the lymphoid organs where they interact with, and activate, B- and T-lymphocytes. In this way, activation of DCs by foreign pathogens can lead to the activation of both the innate and adaptive immune responses, allowing the body to fully respond to the perceived threat[30].

Table 1 Major functions of the innate immune cells in both inflammation and cancer

Inflammatory cell	Immune function	Role in cancer
Neutrophil	Secretion of cytokines/chemokines to modulate other cells in the immune response	Maintenance of pro-angiogenic phenotype[242]
	Release of cytotoxic granules	Suppression of anti-tumor immunity[243]
	Phagocytosis	Promotion of metastasis[244]
Mast cell	Release of cytotoxic granules	Suppression of anti-tumor immunity[245]
	Enhancement of immune cell recruitment	Stimulation of angiogenesis[152]
	Permeabilization of blood vessels	Direct stimulation of cancer cell growth[246]
		Secretion of mitogenic factors[157]
Macrophage	Phagocytosis	
	Promotion of T-lymphocyte activation	
M1	Initiation of immune cell response	
	Antigen presentation	
M2	Wound repair	
	Immunosuppression	
	Tissue remodeling	
	Resolution of immune response	
TAM		Secretion of Arginase-1[19]
		Support of Treg activation[247,248]
		Promotion of angiogenesis[16]
		Enhancement of tumor metastasis[17]
MDSC	Suppression of NKC and T-lymphocyte activation	Production of ROS[22]
		Secretion of peroxynitrite[23]
		Secretion of Arginase-1[24,25]
		Induction of Treg[26]
		Depletion of cysteine[27]
NKC	Release of cytotoxic granules	Tumor cytotoxicity[28]
DC	Antigen presentation	

TAM: Tumor-associated macrophage; MDSC: Myeloid-derived suppressor cell; NKC: Natural killer cell; DC: Dendritic cells.

Adaptive immunity

Innate immunity is a powerful first defense against invading pathogens; however, it is most effective against cells bearing antigens that are common to many pathogens, and can be subverted by quickly evolving cell types, as in the case with cancer cells. The adaptive arm of the immune system is responsible for controlling those pathogens that have overcome the innate response and can provide long-lasting immunity against specific infectious agents.

T- and B-lymphocytes comprise the adaptive arm of the immune response. T-lymphocytes are grouped into classes based on their cell-surface proteins which mediate distinct effector functions. Cytotoxic T-lymphocytes express CD8 and are responsible for killing cells expressing foreign antigen by activating the target cell's apoptosis program, leading to subsequent cell death[30]. Helper T-lymphocytes express CD4 on their surface and assist in the activation of CD8$^+$ T-lymphocytes, B-lymphocytes and macrophages *via* secretion of specific cytokines, thus extending their function across both the innate and adaptive immune responses[30]. A subset of CD4$^+$ lymphocytes, Tregs, protects the body from autoimmune responses. Tregs suppress T-lymphocyte activation in a cytokine independent, cell-contact-dependent manner[31].

B-lymphocytes are one of the main cell types responsible for the body's ability to mount a long-term pathogen-specific response. Each B-lymphocyte produces a single species of antibody, and once activated, proliferates into an antibody-secreting effector cell. It is largely through the action of B-lymphocytes that the body is able to maintain an immunological memory and initiate and immediate response to foreign pathogens it has already encountered[32].

Although inflammation serves to protect the body from harm, it also plays a major role the development of disease, including cancer[33]. Pancreatic cancer, in particular, is heavily influenced by the inflammatory response associated with pancreatic injury and disease. Pancreatitis, or inflammation of the pancreas, is a relatively common condition that often leads to irreversible pancreatic damage and leaves the pancreas vulnerable to the development of neoplastic disease.

PANCREATITIS

The pancreas is comprised of both exocrine (acinar and ductal cells) and endocrine (islets of Langerhans) cells. The exocrine pancreas functions to produce and secrete digestive enzymes into the small intestine whereas the endocrine pancreas is primarily responsible for producing hormones crucial for glucose homeostasis. Dysfunction of the pancreas due to disease or injury can lead to impaired digestion, hypoglycemia and diabetes[34]. Acute pancreatitis (AP) is one of the most commonly diagnosed gastrointestinal diseases, with over 200000 patients admitted to the hospital each year[35]. Patients typically present with acute abdominal pain which may be accompanied by nausea and vomiting, and display increased serum concentrations of amylase and lipase[36]. Pathologically, AP

presents as acinar degranulation, increased occurrences of autophagosomes, formation of dilated acinar lumina and autodigestive fat necrosis[37]. Risk factors for AP include alcohol consumption, gallstones, and smoking, however many cases are idiopathic[38-41]. Although the clinical symptoms of AP often resolve completely, more severe cases can lead to serious complications and even death in a small percentage of patients[42]. Complications associated with AP include pancreatic necrosis[43], infection leading to sepsis[44,45], and systemic inflammatory response syndrome (SIRS) leading to distant organ damage and failure (multiple organ dysfunction syndrome, MODS)[46]. These complications significantly increase the risk of mortality from AP. Additionally, recurrent bouts of AP lead to fibrosis/damage and chronic pancreatitis (CP), a risk factor for the development of pancreatic cancer[4,47].

CP is characterized by acinar loss, extensive fibrosis and immune cell infiltrate, and is a strong risk factor for pancreatic cancer[47-49], which may develop 10-20 years following CP diagnosis[3]. Although CP shares many of the same risk factors, causes, and symptoms as AP, the clinical presentation differs dramatically. Serum levels of pancreatic enzymes amylase and lipase are elevated in AP due to the acute damage caused to the pancreas. In contrast, these enzymes are either normal or only mildly elevated in CP[50]. The chronic inflammation that is the hallmark of CP leads to permanent damage and loss of pancreatic function, leading to diabetes and pancreatic insufficiency[51]. Comprehensive reviews of the diagnosis and etiology of these diseases, as well as factors that distinguish CP from AP, are available[52-54].

Studies from both clinical cases and rodent models of pancreatitis have contributed to the understanding of the inflammatory response to AP and associated syndromes. AP is thought to originate with uncontrolled activation of pancreatic acinar cells and release of digestive enzyme stores leading to autodigestion of the pancreatic cells. This autodigestion releases cellular contents, triggering the recruitment of inflammatory cells. Those inflammatory cells release cytokines and other modulating factors that can amplify the inflammatory response, causing systemic inflammation that can progress to SIRS and MODS. The events responsible for initiating the premature activation of digestive enzymes are not fully understood, but include trypsin auto-digestion[55], generation of reactive oxygen species, disturbances in microcirculation[56], calcium overload, and leukocyte overstimulation[57], and are reviewed in detail elsewhere[58-61].

There is a close relationship between the inflammatory response to AP and clinical severity of the disease[62]. Indeed, overstimulation of leukocytes, specifically neutrophils, results in systemic activation and is thought to be a major cause for severe AP-associated death[57,63]. Many animal models for AP exist in which pancreatitis is induced by pancreatic injury or surgical blockage. While insights into the possible mechanisms of the immune response in the pancreatic disease process can be gained from these models, they do not exactly replicate the clinical disease. Additionally, it should be noted that whereas rodents are the model of choice to study the immune system and its interaction with organ systems, significant differences exist between the human and rodent immune systems in the balance of leukocyte subsets, and in the expression of inflammatory mediators, cytokines and cytokine receptors, as well as the significantly different immunological environments occupied by either species[64]. For these reasons, animal models of pancreatitis will be addressed separately in this section of the review.

Inflammatory response to AP: Clinical findings

AP is characterized by early recruitment and activation of polymorphonuclear cells, the majority of which are neutrophils[57]. Activation of neutrophils can be identified by rising levels of serum neutrophil elastase in the early course of AP, typically within the first two days of diagnosis[62,65,66]. This is accompanied by the detection of metabolically hyperactive granulocytes in the pancreatic tissue of AP patients within 48 h of admission to the hospital, suggesting that granulocyte activation is an early AP event[67]. Neutrophil recruitment is followed by recruitment of the macrophage-monocyte system in the subsequent 2-3 d, as determined by rising levels of C-reactive protein (CRP) in the serum[62,68]. As elastase and CRP are released by neutrophils and macrophages, respectively, the presence of these proteins in the blood of pancreatitis patients is used as an indirect indicator of the recruitment and activation of these inflammatory cells in the pancreas.

Complications associated with AP can be grouped into two phases: immune overactivation and immune suppression. In the first phase, control is lost over the local inflammatory response, leading to excessive and uncontrolled systemic activation of inflammatory cells and mediators[69]. This often leads to the development of SIRS and MODS, and is associated with death within one week of disease[70]. In a subset of patients, the body responds to systemic inflammation with compensatory anti-inflammatory response syndrome (CARS)[71]. CARS initiates the second phase of complications associated with AP and can lead to immune deficiency or suppression, rendering the body susceptible to infection. These patients go on to develop AP-associated infections[44] that are associated with excessive CARS.

Systemically, a decrease in circulating lymphocytes, including B- and T-lymphocytes (both CD4^{+} and CD8^{+}), as well as NKCs, is often seen in AP[67,72,73]. A decrease in circulating lymphocytes is associated with more severe disease and is often predictive of AP-associated systemic infection[74-76]. Kylänpää-Bäck *et al*[77] demonstrated a significant decrease in monocyte surface expression of human leukocyte antigen-DR (HLA-DR), a hallmark for systemic immunosuppression, in the first 24-48 h following AP diagnosis. Many independent studies have confirmed that a decrease in serum lymphocytes, as well as monocyte HLA-DR, correlates with more severe disease and increased mortality[73-75,77,78].

It is important to understand that the clinical timeline of inflammatory response to AP is a relative concept, as
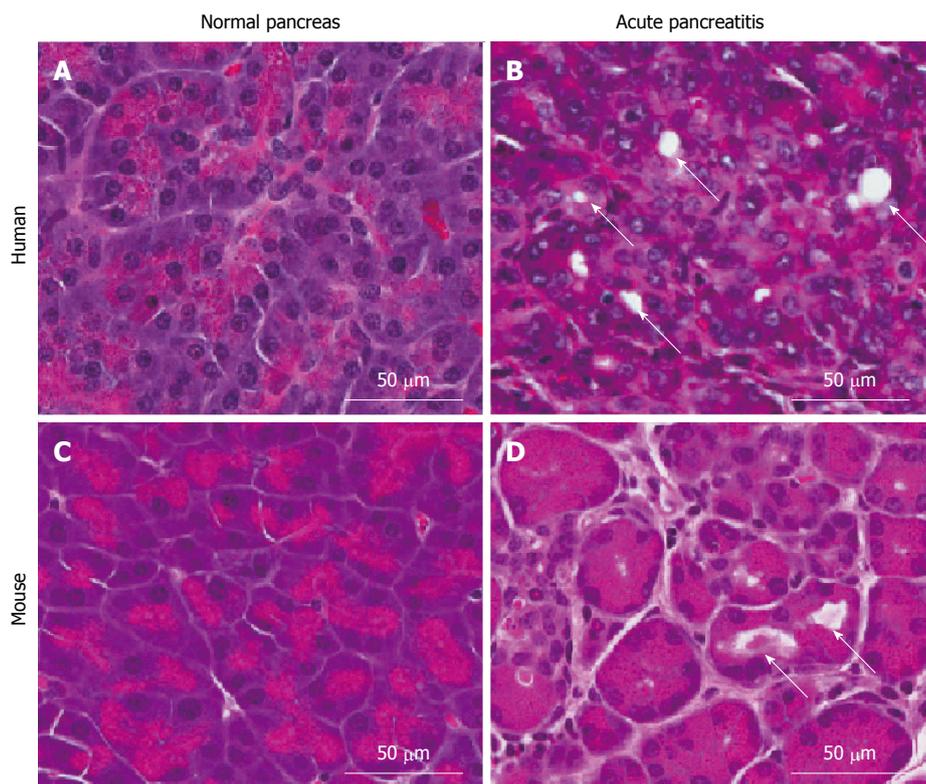
Figure 1 Comparison of histology of human and mouse pancreatic tissue. A: Normal human pancreas; B: Human acute pancreatitis (AP); C: Normal mouse pancreas; D: Mouse AP. Arrows indicate acini with dilated lumina, as commonly seen in AP.

data is dependent on the timing of the patient's admission to the hospital. Additionally, as biopsies are not routinely performed on AP patients, a detailed histological analysis of the pancreatic tissue is often not available. It is these limitations of clinical analysis that led to the use of rodent models in an attempt to more fully understand the mechanism of disease initiation and progression.

Animal models of AP

Several animal models have been developed to study the complex interactions that occur between the pancreatic epithelium and the many inflammatory cell types recruited to the pancreas. These models can be used to more precisely map the time course of the inflammatory response in AP, as well as to determine the mechanism(s) of damage and recovery in order to identify potential therapeutic targets. Although many species have been used to evaluate AP, this review will focus on data generated using rodents. Rodent models are used most often because of their cost-effectiveness and ease of characterization and genetic manipulation; however, no one model completely recapitulates all components of human disease.

The most common method of modeling pancreatitis in rodents is secretagogue hyperstimulation leading to premature intrapancreatic activation of digestive proteases. In this model, administration of high concentrations of the intestinal hormone cholecystokinin, or its molecular ortholog, caerulein, leads to autodigestion of the pancreas[79] and pancreatitis-like pathology including vacuolization, edema, acinar degranulation, dilated acinar

lumina, necrosis, lung injury, and cytoplasmic destruction of pancreatic acini[80,81]. Figure 1 shows the histological similarities between human AP and caerulein-induced rodent AP. Another model for AP is surgical ligation of the pancreatic duct. Although this method was designed to mimic clinical gallstone-induced pancreatitis, it often produces a milder form of the disease and is more technically demanding and invasive[82]. Other models of AP include administration of high concentration of L-arginine leading to acinar necrosis[83], feeding of a choline-deficient diet, leading to severe necrotizing pancreatitis[84,85], and overstimulation of the immune system using bacteria or toxins[86].

Inflammatory response to AP: Animal models

Animal models of AP allow histological analysis of all stages of the disease, providing much information regarding the pathogenesis of AP. Although induced AP can present differently depending on animal model utilized, nearly all models result in a recruitment of neutrophils within hours of treatment (Figure 2), thus confirming neutrophils as one of the first responders to pancreatic damage[81,87-91]. Neutrophils have been shown to mediate systemic remote organ injury and death in a murine model of hemorrhagic pancreatitis[92]. Significant macrophage infiltration to the pancreas is observed shortly after caerulein-induced AP (Figure 2), and macrophage-derived macrophage-inflammatory protein-2 (MIP-2) is known to play a role in progression of AP through attraction of leukocytes, promoting tissue injury[93]. Therefore, results
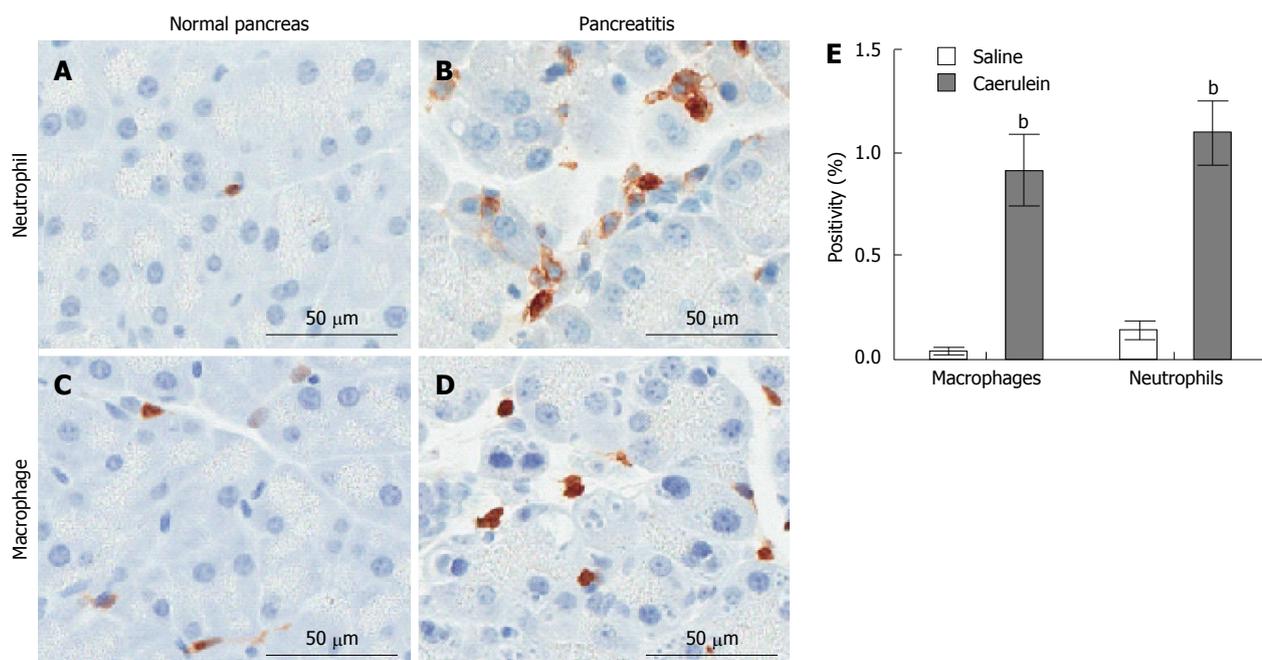
Figure 2 Induction of innate immune response in a mouse model of acute pancreatitis. Mice were injected intraperitoneally with 50 µg/kg caerulein or saline hourly for 7 h (Mayo Clinic IACUC protocol A48510). The pancreas was isolated one hour following the last injection and analyzed immunohistochemically for the presence of neutrophils (Ly6B.2: AbSerotec) or macrophages (Mac-2; Cedarlane Diagnostics). A: Neutrophil infiltration in saline-treated mice; B: Neutrophil infiltration in caerulein-treated mice; C: Macrophage infiltration in saline-treated mice; D: Macrophage infiltration in cearulein-treated mice; E: Stained slides were imaged using ScanScope XT (Aperio, Vista, California) and immunohistochemical staining was quantified using the Aperio ImageScope reader. Mean ± SD is plotted for each group of *n* ⩾ 4 mice, [b]*P* < 0.01 *vs* saline group.

from caerulein-treated mice are consistent with clinical data that macrophages also contribute to the early inflammatory response to AP[93].

Mast cells are present in the normal pancreas and undergo degranulation upon induction of AP[87,94]. Inhibition of mast cell degranulation decreases pancreatic inflammation without affecting pancreatic damage[94], suggesting that mast cells primarily play a role in releasing or activating additional inflammatory mediators. DCs are rare in the normal pancreas, but pancreata of caerulein-treated mice exhibit a significant increase in mature DCs. These DCs are crucial for pancreatic viability during injury, as their depletion during caerulein- and L-arginine-induced AP leads to massive pancreatic cell death[95].

A distinct decrease in B- and T-lymphocytes is seen in serum from patients with AP, suggesting a systemic inhibition of the immune system. In support of impaired cell-mediated immunity, interleukin (IL)-2, a product of T-lymphocytes, is decreased in mononuclear splenic cells in a murine model of AP[96]. However, it has been theorized that decreases in circulating lymphocytes are not due to immune suppression but instead to a redistribution of lymphocytes from the blood pool to the pancreas[67]. This concept is difficult to test clinically, as most AP patients will not undergo pancreatic biopsy. In support of this theory, Demols *et al*[97] demonstrated that T-lymphocytes, specifically CD4^{+} cells, are increased in the murine pancreas following induced AP. This study showed that T-lymphocytes have a role in mediating tissue injury, as depletion of CD4^{+} T-lymphocytes, or elimination of T-lymphocytes using genetic models, reduced the severity of AP, and this injury was reversible by a T-lymphocyte transfer[97].

Role of cytokines in AP

AP is characterized by excessive recruitment and activation of leukocytes within the pancreas, and in many cases, other organs. Recently, a theory has emerged that the damaged pancreatic epithelium itself is responsible for expression of the first wave of inflammatory mediators, effectively launching the inflammatory cascade that leads to recruitment and activation of immune cells. This is supported by the fact that acinar cells are capable of producing a number of inflammatory mediators in response to damage or noxious stimuli[98-100]. When activated or stressed, isolated acinar cells have been shown to express cytokines[101-104] and chemokines[105] (Table 2 and Figure 3). Detailed reviews regarding the major roles of these mediators in AP and associated conditions are available[106-108]. In addition, activated immune cells secrete numerous chemotactic factors which are capable of perpetuating the immune cell activation cascade.

IL-1β and tumor necrosis factor (TNF) α production by pancreatic cells is thought to be a relatively early event in the activation of the cytokine cascade in AP, with IL-6 secretion occurring later in the disease process[109]. Serum IL-6 correlates with disease severity in humans[110,111] and is useful as an early diagnostic marker of AP[112,113]. In a rat model of pancreatitis, TNFα levels were shown to rise over time following induction of pancreatitis[114], and neutralization of TNFα *via* antibody improved all aspects of pancreatitis (elevated serum amylase, hematocrit, ascites) supporting an important role for TNFα in the
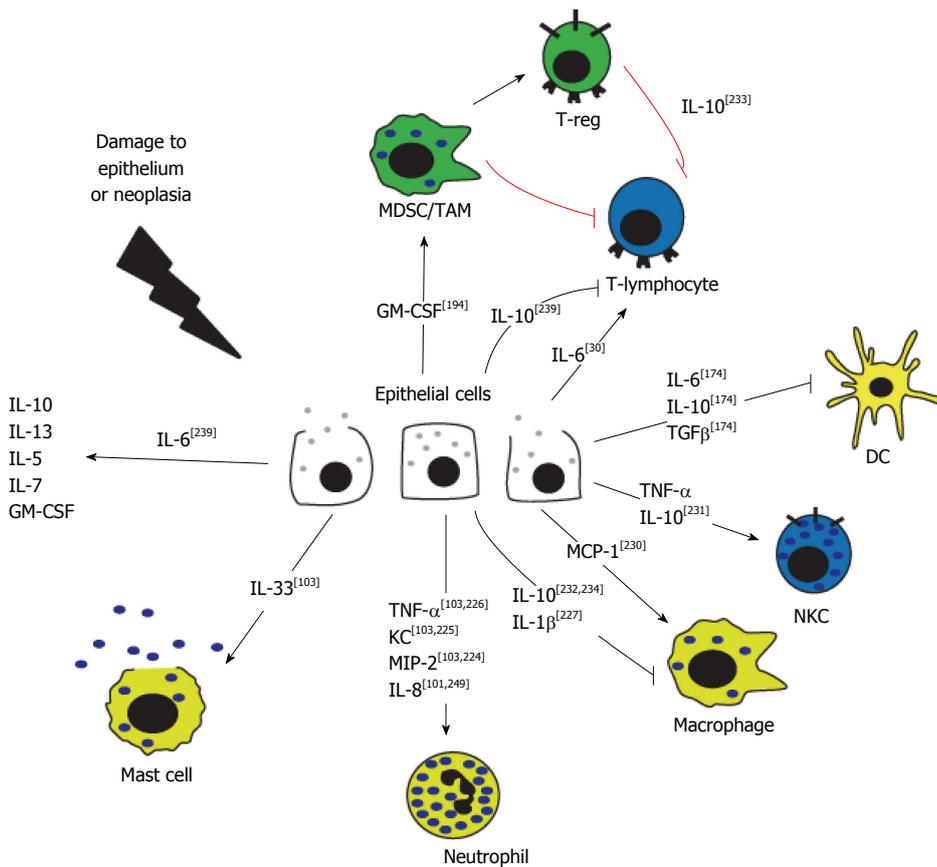
Figure 3 Cytokine signaling in the pancreatic epithelium. In response to damage or disease, the pancreatic epithelial cells release cytokines and chemokines. Activated immune cells secrete numerous chemotactic factors in response to, and in addition to, those secreted by the epithelial cells. TNF: Tumor necrosis factor; IL: Interleukin; MDSC: Myeloid-derived suppressor cells; TAM: Tumor-associated macrophage.

Table 2 Cytokines released during pancreatic injury

Cytokine	Expressed by	Acts on
GM-CSF	Acini[194]	Neutrophils[231], MDSCs[194]
IL-1β	Acini[104,238], activated macrophages[237], neutrophils[236]	Macrohpage (inhibition)[227]
IL-6	Acini[239], monocytes[30], macrophages[30], endothelial cells[30] fibroblasts[30], smooth muscle cells[30], IL-1b[30], TNFα[30]	CRP[228,229], T-lymphocyte[30], DCs (inhibition)[174]
IL-8	Acini[105], IL-1[241], TNFα[241], macrophages[241], neutrophils[235]	Neutrophils[223]
IL-10	Acini[240], Treg[233]	T-lymphocytes (inhibition)[233], DCs (inhibition)[174], macrophages (inhibition)[232,234]
IL-12	Activated macrophages[231], dendritic cells[30]	NKCs[231]
IL-33	Acini[105]	Mast cells[103]
KC	Acini[105], monocytes and neutrophils[250]	Neutrophils[225]
MCP-1	Acini[105]	Macrophages[230]
MIP-2	Acini[105], activated macrophages and neutrophils[93]	Neutrophils[224]
TNFα	Acini[105], activated macrophages[99], neutrophils[236], mast cells[226]	Neutrophils[226], NKC[231]

IL: Interleukin; MCP-1: Monocyte chemotactic protein-1; MIP-2: Macrophage inflammatory protein 2; TNFα: Tumor necrosis factor-α; MDSC: Myeloid-derived suppressor cell; NKC: Natural killer cell; DC: Dendritic cells.

pathogenesis of AP[114]. Blockade of IL-1 signaling using IL-1 receptor antagonist (IL-1ra) attenuates the rise in both IL-6 and TNFα, as well as lessens pancreatic damage in the context of AP, supporting early expression of IL-1 and confirming its role as an important mediator for subsequent cytokines[99].

IL-8 is another cytokine implicated in the early stages of the disease process. IL-8 is an established secretory product of activated macrophages[115], but has also been detected in the pancreatic epithelial cells of clinical and pre-clinical pancreatitis tissue[101,102], suggesting a damaged pancreatic epithelium as a possible source of IL-8. Gross *et al*[116] determined that IL-8 in the serum of pancreatitis patients correlates with disease severity, and showed a significant positive correlation between serum IL-8 and neutrophil elastase, a marker of neutrophils found upregulated in the early stages of AP. As IL-8 is a potent neutrophil activator[117], infiltration of neutrophils may be initiated by
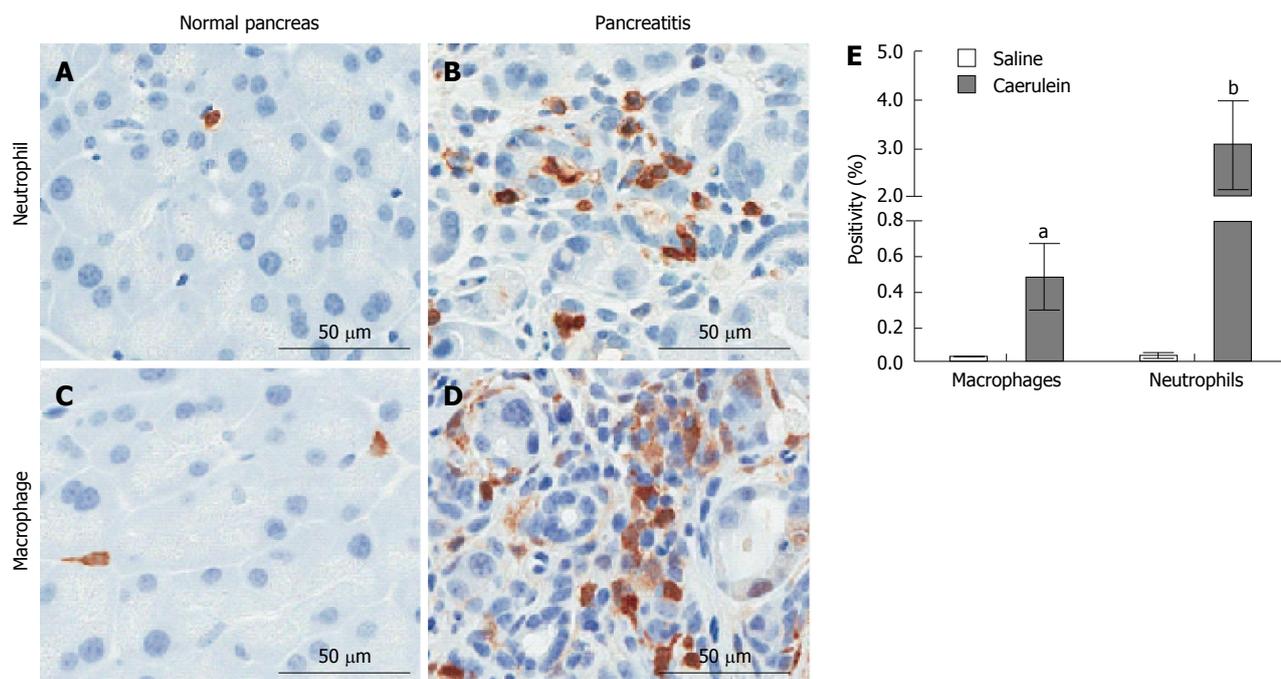
Figure 4 Induction of innate immune response in a mouse model of chronic pancreatitis. Mice were injected intraperatoneally with 250 μg/kg caerulein or saline twice daily, six days per week for two weeks (Mayo Clinic IACUC protocol A48510). The pancreas was isolated 24 h following the last injection and analyzed immunohistochemically for the presence of neutrophils (Ly6B.2: AbSerotec) or macrophages (Mac-2; Cedarlane Diagnostics). A: Neutrophil infiltration in saline-treated mice; B: Neutrophil infiltration in caerulein-treated mice; C: Macrophage infiltration in saline-treated mice; D: Macrophage infiltration in cearulein-treated mice; E: Stained slides were imaged using ScanScope XT (Aperio, Vista, California) and staining was quantified using the Aperio ImageScope reader. [a]*P* < 0.05, [b]*P* < 0.01 *vs* corresponding saline treated control. Mean ± SD is plotted for each group of *n* ≥ 5 mice.

secretion of IL-8 from the pancreatic parenchyma. Resident and infiltrating macrophages can also secrete IL-8, recruiting more neutrophils and effectively reinforcing the cycle of inflammation.

Based on clinical studies and analyses of murine models of AP, our knowledge of the inflammatory events in AP can be summarized as: Damage to the pancreas, either caused by, and/or resulting in pancreatic autodigestion, leads to the release of inflammatory mediators from acinar cells. Many of these mediators are potent neutrophil attractants and are likely responsible for neutrophil recruitment to the pancreas as the first wave of inflammatory response. Once in the pancreas, activated neutrophils secrete additional cytokines, thus amplifying the inflammatory response by recruiting additional neutrophils as well as macrophages and other cells of the innate immune response. These cell types are often able to resolve the damage to the pancreas, with limited activation of the adaptive immune response. However, recurrent bouts of AP can lead to a much more serious condition, CP, which presents as a significant risk factor for the development of pancreatic cancer.

Whereas AP is often a self-limiting condition, CP is defined as longstanding inflammation of the pancreas that leads to progressive and irreversible changes. Clinically, CP is characterized by macrophage and T- and B-lymphocyte infiltration into the pancreas[118-122], although peripheral T-lymphocytes appear to decrease[123,124]. Infiltrating mast cells have also been described in the pancreas of CP patients[120,125,126], positively associating with pancreatic fibrosis[126] and pain[120,125]. Finally, immunosuppressive Tregs have been identified in the bone marrow, blood and lesions of CP patients[127].

In general, animal models of CP are generated by repeated induction of AP[128]. In a rat model of CP, both macrophages and CD8+ T-lymphocytes were prevalent in the connective tissue and parenchyma, and CD8+ T cells infiltrated the pancreatic lobules[118]. In a mouse model of CP, a significant increase in both macrophages and neutrophils were observed in response to caerulein (Figure 4), and depletion of these inflammatory cell types significantly reduced pancreatic injury as determined by serum amylase release, and pancreatic lesion formation and fibrosis[129,130]. Similar to clinical findings, mast cells were found to play a significant role in the pathogenesis of pain in a mouse model of CP[125]. Although a serious disease on its own, CP is also a significant risk factor for PDAC and may represent a condition that promotes PDAC development[131].

PANCREATIC CANCER

PDAC is the most common form of pancreatic cancer. PDAC is thought to develop by progression through a distinct series of pre-cancerous stages, pancreatic intraepithelial neoplasias (PanINs), before advancing to adenocarcinoma[132]. Greater than 90% of all pancreatic adenocarcinomas contain an activating mutation in codon 12 of the Kirsten rat sarcoma viral oncogene homolog (Kras)[133]. This mutation is thought to occur early in
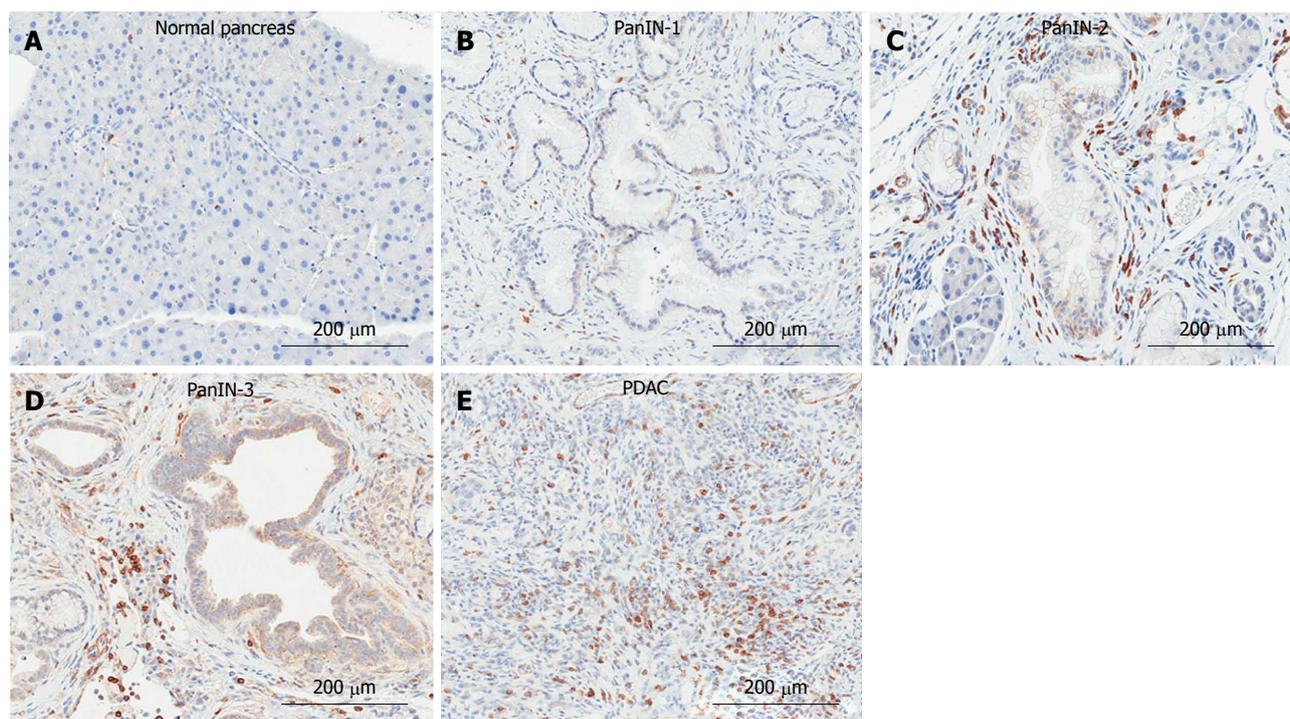
Figure 5 Immunohistochemical detection of T-lymphocytes in the Kras[G12D]-induced mouse model of pancreatic ductal adenocarcinoma. A: Normal pancreas; B: Pancreatic intraepithelial neoplasia (PanIN)-1; C: PanIN-2; D: PanIN-3; E: Pancreatic ductal adenocarcinoma (PDAC) tissue was analyzed by immunohistochemistry for the presence of T-lymphocytes (CD3: abcam).

the disease process and drive initiation and progression of PDAC. Indeed, Kras mutations are prevalent even in early stage PanINs and their presence correlates with disease progression[134]. In a classic PDAC mouse model, tissue specific expression of oncogenic Kras[G12D] from the endogenous Kras promoter (Lox-Stop-Lox- Kras[G12D]; Pdx-1-cre, KC mouse model) in the mouse pancreatic epithelium recapitulates the full spectrum of human PDAC, including development of PanINs, with progression to adenocarcinoma at approximately 1-2 years of age[135]. For this reason, Kras[G12D]-expressing mouse models are commonly used to study the initiation and development of PDAC.

It is well established that inflammation plays a major role in the development and progression of pancreatic cancer. Chronic inflammation of the pancreas (pancreatitis) is a significant risk factor for development of PDAC, and PDAC itself is characterized by marked leukocyte infiltration[4,47,48]. Notably, multiple immunosuppressive cell types are observed in pancreatic cancer tissue, suggesting a dysfunction of the immune response, likely mediated by the cancer itself (as described below). Immune dysfunction in PDAC is typified by (1) the recruitment and activation of immunosuppressive cell types; (2) the presence of tumor-supportive immune cells; and (3) a lack of immunity due to defective or absent immune cells. Clinical data and experimental rodent models contribute to the current understanding of the inflammatory response to PDAC as well as the impairment of that response that is a common evasion tool of most cancers.

Immunosuppression in PDAC

Clinical and murine evidence support the existence of profound immunosuppression in PDAC tissues. Clark *et al*[6] characterized the immune cell influx in various stages of PDAC ranging from normal pancreas to PanINs to invasive carcinoma using the KC mouse model of pancreatic cancer. The study describes an early immunosuppressive phenotype in pancreatic cancer, challenging the classic immunoediting "elimination" phase and suggesting that tumor cells "escape" from immune control almost immediately. At the early PanIN stages, Tregs and MDSCs dominate the immune infiltrate. As the disease progresses to PDAC, CD4^{+} and CD8^{+} cells are inconsistently found associated with the tumor, and those CD8^{+} cells associated with the tumor lack evidence of activation, suggesting a suppressed immune environment[6]. In all stages of disease, there is a strong inverse correlation between MDSCs and CD8^{+} T-lymphocytes, suggesting that MDSCs are a mediator of tumor immunosuppression[6].

Clinically, lymphocytes are prevalent in pancreatic cancer. CD8^{+} cells are elevated in the circulation of PDAC patients[136], and leukocytes, the majority of which are T-lymphocytes, surround the pancreatic lesion[137]. T-lymphocytes are found more frequently in the fibrotic interstitial tissue than in the intraepithelial area of the pancreatic cancer[138], and distribute heterogeneously within the tissue, presenting as both scattered cells and focal areas of high accumulation[139]. An example of T-lymphocyte accumulation in progressive stages of the KC model of PDAC is shown in Figure 5.

The majority of the T-lymphocytes in PDAC are

CD4^{+} Tregs, supporting an immunosuppressive phenotype. Tregs are significantly increased in the blood of PDAC patients as well as in the pancreatic tissue[140]. They are found typically in the stromal areas of the tumor, and only occasionally in association with tumor epithelial cells[141]. Hiraoka *et al*[141] examined clinical samples of pre-malignant lesions and found that Treg accumulation correlates with the progression of both of the major preneoplastic lesions in pancreatic cancer, PanINs and intraductal papillary mucinous neoplasms (IPMN). The association of Tregs with IPMN progression has been independently confirmed[142]. Additionally, Tregs correlate with metastasis[143] and tumor grade, and negatively correlate with patient survival[141].

Treg infiltration in the development of pancreatic cancer is confirmed in murine models of PDAC. A significant accumulation of Tregs is found in the KC model of PDAC[6]. In another murine tumor model, subcutaneous injection of mouse pancreatic tumor cells into syngeneic mice results in a significant increase in Tregs in the spleen and tumor-draining lymph nodes of these mice[144]. Tan *et al*[145] have shown in both human PDAC and a murine model of pancreatic cancer that tumor cells produce elevated levels of ligands for the CCR5 receptor, a receptor preferentially expressed by Tregs. Interruption of this receptor-ligand signaling reduces tumor size, as well as Treg recruitment to that tumor, suggesting Tregs are likely recruited in response to direct signaling from the tumor cells.

MDSCs are another immunosuppressive cell type prevalent in PDAC that contributes to immune dysfunction. Whereas MDSCs are absent in the healthy human pancreas, they are readily detected in the stroma of PDAC, comprising approximately 67% of the infiltrating leukocytes[146]. MDSCs are also found in the blood and bone marrow of PDAC patients, and are significantly higher in cases of metastatic disease compared to patients with local tumor[146-148], supporting a previous report that MDSC count correlates with disease stage[149]. Additionally, circulating MDSC numbers are found to be an independent prognostic factor for survival[147].

The functional role of MDSCs in tumor promotion has been characterized in murine models of PDAC[146,147,150]. Following subcutaneous injection of the non-metastatic pancreatic cancer cell line, Pan02, MDSC infiltration is detected in bone marrow, spleen, and tumor[146]. This increase is associated with decreased levels of CD4 and CD8 and increased levels of Tregs in circulation. These MDSCs are able to suppress CD8^{+} T-lymphocytes *in vitro* and promote initial tumor growth when co-injected with Pan02[146]. Similarly, in a spontaneous murine model of tumor formation [driven by pancreas-specific overexpression of transforming growth factor (TGF) α and loss of Trp53], MDSC numbers increase in the lymph nodes, blood and pancreas as early as the pre-malignant lesion stage, and increase further upon tumor development. *In vitro*, these tumor-associated MDSCs were shown to possess arginase activity and suppress T-lymphocyte

responses[151]. In the KPC model of metastatic pancreatic cancer (driven by pancreas-specific expression of oncogenic Kras[G12D] and mutant Trp53[R172H]), MDSCs accumulate in tumor and spleen and comprise 20%-30% of all leukocytes. MDSCs are closely associated with tumor cells and metastases, suppress proliferation of T-lymphocytes, and express high levels of arginase and nitrite upon stimulation[150]. Collectively, these animal models strongly support a role for MDSCs in tumor promotion through T-lymphocyte inhibition and resulting immunosuppression.

To summarize, both clinical and animal models provide strong evidence for accumulation of immunosuppressive cells types, and subsequent inhibition of immune function in PDAC. The consequence of a suppressed immune system is not direct tumor promotion, but rather alleviation of an important barrier to tumor growth. As described by the cancer immunoediting model, a functional immune system can act as an extrinsic tumor suppressor, identifying and eliminating tumor cells[10]. Alterations in tumor immunogenicity or suppression of the immune response are crucial mechanisms by which this barrier is surmounted, allowing the cancer to progress.

Tumor-supportive immune cells

In addition to the immunosuppressive cell types that inhibit effector T-lymphocyte immunity, other immune cells adopt a tumor-supportive role in the context of PDAC. Once a tumor is established, factors secreted by effector immune cells can often promote, rather than prevent, tumor progression. Mast cells accumulate in PDAC tissue and, along with macrophages, express tumor-promoting factors including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)[152]. VEGF and FGF have been shown to stimulate the growth of pancreatic tumors and maintain blood vessels[153,154]. Mast cell accumulation correlates with higher tumor grade, worse survival[155], lymphatic and microvascular invasion[156], and lymph node metastasis[152,156]. In support of the idea that mast cells can support tumor growth, *in vitro* analyses demonstrate that mast cell-derived factors can promote PDAC cell migration, proliferation, and invasion[155,157]. Interestingly, the importance of mast cells in PDAC appears to be zone specific, with the presence of mast cells in the intratumoral border zone, and not in the intratumoral center or peritumoral zones, correlating with microvessel proliferation, lymph node metastases, and lymphatic and microvascular invasion[156]. Additionally, mast cell accumulation within the intratumoral border is an independent prognostic factor for survival[156]. The significance of mast cell localization likely stems from the ability to establish a pro-tumor microenvironment by degrading tissue surrounding the tumor to promote invasion and by remodeling blood vessels[156]. Taken together, preclinical PDAC models support a role for mast cells in tumor growth and for mast cell accumulation and function at the tumor border[158].

Inflammation can drive the early stages of pancreatic

lesion formation in mouse models[159]. A recent study demonstrated that two macrophage secreted inflammatory cytokines could mediate the effects of infiltrating macrophage on acinar cell metaplasia[5]. Matrix metalloproteinase (MMP)-9 and RANTES (regulated on activation, normal T cell expressed and secreted), secreted by pancreas-infiltrating macrophages, are implicated in driving the very first stages of pancreatic lesion formation in a mouse model of cearulein-induced pancreatitis[5].

Immune factors that are responsible for pathogen killing can also promote tumor cell migration and metastasis[160]. Additionally, tumor-promoting immune factors can promote tumor cells to adopt a more fibroblast-like morphology[155,157,160-162] and remodel the tumor environment[156,163] to facilitate migration. In these ways, neutrophil- and B-lymphocyte-derived proteins are implicated in PDAC invasion[160-163]. Neutrophil-derived elastase has been shown to mediate epithelial-mesenchymal transition (EMT) of PDAC cells *in vitro*[161,162]. Neutrophil-derived MMP-9 promotes PDAC tumor growth and angiogenesis[163], and MMP-9-producing neutrophils have been identified at the leading edge of PDAC in a murine model of pancreatic cancer as well at the invasive front of PDAC metastases[164]. Neutrophils, typically one of the body's first lines of defense, are not prevalent in PDAC epithelia[165]. However, they are associated with the predominant stromal component of PDAC, and several reports describe a significant increase in neutrophils when measuring levels in both PDAC tissue and stroma together[162,166]. In PDAC, neutrophils are associated with micropapillary carcinoma of the pancreas and correlate with poor prognosis[165,167]. Although density of neutrophil infiltrate does not correlate with tumor clinical stage[162], a patient's neutrophil: lymphocyte ratio is an independent prognostic indicator of survival following resection of PDAC tumor[168,169].

B-lymphocytes are also able to promote the tumor phenotype by secretion of B-lymphocyte activating factor (BAFF). Koizumi *et al*[160] described *in vitro* data demonstrating that BAFF mediates EMT, increases invasion and promotes motility of PDAC cells, supporting a role for B-lymphocyte-secreted BAFF tumor progression and metastasis. Additionally, BAFF-expressing B-lymphocytes surround and infiltrate tumor cells in clinical samples of PDAC, correlating with increased serum levels of BAFF[160].

Defective immune cells in PDAC

DCs are the link between innate and adaptive immunity, recognizing the presence of foreign pathogens and alerting the adaptive immune cells *via* antigen presentation. However, in a tumor environment, DCs often display an immature phenotype and are defective in their antigen-presenting abilities[170-173]. Tumors express various molecules which are thought to repress DC maturation[174] including IL-10[175,176], IL-4[177], VEGF[178], TGF-β[179], IL-6[180,181], and macrophage-colony stimulating factor[181]. Muc-1, a protein highly expressed on PanIN cells pro-

foundly affects the maturation of DCs. Monti *et al*[182] demonstrated that DCs exposed to tumor mucins do not fully mature and are characterized by a tolerogenic/regulatory cytokine profile, expressing high levels of IL-10 and low levels of IL-12. Peripherally, circulating DCs are significantly decreased in patients with PDAC[183-186], and demonstrate an impaired ability to stimulate T-lymphocyte proliferation[184].

Absent immune cells in PDAC

Similar to chronic pancreatitis, few NKCs are found in PDAC tissue[138]. The basal systemic NKC activity is decreased in PDAC patients, as well as the NKC response to interferon-α, a potent enhancer of NKC activity[187,188]. Decreased NKC activity has been linked to poor patient prognosis[188] and may be indicative of a suppressed innate immune response.

A role for epithelial cells in dysfunction of the immune response

Several lines of evidence point to a strong role for the neoplastic pancreatic epithelial cells in establishing a dysfunctional immune environment. As previously mentioned, PDAC cells can recruit Tregs[145], promote mast cell migration and activation[157], and repress DC maturation[182]. *In vitro*, human PDAC cells inhibit T-lymphocyte proliferation and migration, and this is accompanied by an increase in immunosuppressive cytokines including IL-8 and TGFβ[189]. Immunosuppressive compounds TGFβ and IL-10 are upregulated in PDAC and pancreatitis patient sera[190]. Finally, Muc-1, a mucin highly expressed in PanIN-1 early lesions, can suppress T-lymphocyte proliferation[191,192] and it has been suggested that Muc-1 is responsible for tumor escape from recognition and destruction by immune cells[193].

Murine models of PDAC provide additional support for the role of pancreatic epithelial cells in maintaining an immunosuppressive tumor environment. In the KC mouse model, Kras[G12D]-dependent upregulation of granulocyte macrophage-colony stimulating factor (GM-CSF) is detected in PanINs, and results in recruitment of MDSCs and concomitant inhibition of CD8^{+} T-lymphocytes[194]. This data is supported by the work of Bayne *et al*[150] who describe a model in which tumor-derived GM-CSF recruits myeloid inflammatory cells resulting in the negative regulation of CD8^{+} T cells.

Interaction(s) between immune cells and stroma in PDAC

The development of PDAC is marked by increasing desmoplasia, resulting in the development of a vast stroma that often equals or exceeds the epithelial component of the tumor. The stroma is composed of extracellular matrix proteins and contains various non-epithelial cell types including stellate cells, endothelial cells, and immune cells, but the majority of the stromal cells are activated pancreatic stellate cells (PSCs) and fibroblasts[195]. Activated PSCs promote cancer cell growth and immune cell dysfunction

in PDAC[196,197]. PSCs have been implicated in promoting transformed growth, cellular invasion and EMT of PDAC cells, as well as in promoting PDAC tumor incidence, size, and metastasis in an orthotopic mouse model[198-200].

PSCs can have profound effects on the immune cell milieu of PDAC, and have been shown to express a number of growth factors and cytokines[201]. Inflammatory cells recruited to the tumor site are most often found in the stroma rather than infiltrating the epithelial cells[202]. Indeed, activated PSCs have been shown to attract and adhere to CD8^{+} T-lymphocytes, sequestering them in a juxtatumoral compartment (< 100 μm from tumor) and preventing their access to PDAC tumor cells[197]. One report showed that 94% of tumor-associated T-lymphocytes were either inactivated or did not make it to tumor because they were trapped in the tumor stroma[190]. A potential mechanism by which PSCs may regulate T-lymphocyte trafficking in PDAC is *via* CXCL12 expression, as PDAC T-lymphocytes express elevated levels of the CXCL12 receptor[197]. Additionally, activated PSCs express a number of cytokines and adhesion-mediating molecules[203], and have been shown to produce MDSC-promoting cytokines, including IL-6, M-CSF, and VEGF, and to promote differentiation of MDSCs from peripheral blood mononuclear cells[204]. Finally, PSCs stimulate mast cell activation, and, conversely, mast cell-derived factors can stimulate PSC proliferation[157]. These data suggest that not only can activated stellate cells directly promote the cancer cell phenotype, but they also contribute to the immunosuppressive phenotype that characterizes PDAC and hampers immunotherapy efforts.

Cancer stem cells

In addition to the immune dysfunction that is prevalent in PDAC, new evidence is emerging to support a role for immune-cytokines in promoting cancer metastasis *via* interaction with cancer stem cells (CSCs). CSCs are characterized by their ability to self-renew, capability to develop into multiple lineages, enhanced tumor-initiating ability, and resistance to typical cancer therapies[205]. CSCs are thought to be responsible for cancer progression, resistance to standard therapies and tumor relapse. Specifically, pancreatic CSCs have been shown to play a crucial role in the aggressive nature of pancreatic cancer and the resistance to therapy that is a hallmark of this cancer (reviewed in Dorado *et al*[206]). They share an intimate relationship with the tumor microenvironment, regulating, and being regulated by, cells present therein[207,208]. Specifically, TAMs regulate CSC tumorigenicity and anticancer drug resistance through production of specific growth factors[208]. Additionally, inflammatory cytokines play a role in mediating CSC self-renewal[209].

Recent work suggests that inflammatory cells can promote dissemination of pancreatic CSCs. Rhim *et al*[210] reported that pancreatic cancer cells exhibiting cancer stem cell properties, including enhanced tumor-initiating capacity, survival, and self-renewal, left the pancreas and were detected in the circulation at the immediate early

stages of pancreatic cancer development (PanIN formation), prior to overt tumor formation. The presence of CSCs in the circulation was significantly elevated following induction of pancreatitis, and conversely, treatment with an anti-inflammatory drug, dexamethasone, resulted in a significant decrease in the circulating CSCs. These data support a role for inflammation in promoting dissemination of pancreatic CSCs and potentially in PDAC metastasis.

THERAPY FOR PDAC

Immunotherapy, therapeutic modulation of the immune response, has emerged as a promising line of treatment for many cancers including PDAC[211,212]. Types of immunotherapy that are currently being tested in clinical trials for pancreatic cancer include whole cell, peptide/DNA, antigen pulsed DC, and monoclonal antibody vaccines. Whole cell vaccines typically use irradiated pancreatic cancer cells as the immunogen. These cells have the potential to elicit a robust immune response because they express the full complement of tumor-associated antigens. There have been significant survival advantages reported in resected pancreatic cancer patients using whole cell vaccines such as Algenpantucel-L, an irradiated, live combination of two allogenic pancreatic cell lines[213]. Vaccines comprised of peptides or DNA corresponding to tumor antigens are designed to enhance the cytotoxic T-lymphocyte response. Peptides corresponding to oncogenic Ras, telomerase, VEG-F receptor, carcinoembryonic antigen (CEA), survivin, and Muc-1 have all been successful at prolonging life in pancreatic cancer patients in clinical trials[211,213].

Antigen-pulsed DC vaccines take advantage of the antigen-presenting abilities of DCs to elicit a robust adaptive immune response specific to a tumor antigen of choice[214]. DCs pulsed with Muc-1 and CEA antigens have both been used in clinical trials for pancreatic cancer[215]. Finally, monoclonal antibodies against cell surface tumor antigens are used to induce antibody-dependent cell cytotoxicity[211]. Clinical trials have evaluated antibodies against mesothelin, CEA, and epidermal growth factor receptor for pancreatic cancer treatment[211,215]. Of importance, the success of an anti-cancer vaccine relies on its ability to elicit an immune response in the host, and thus may not exhibit uniform effectiveness in all patients depending on the ability of their immune system to generate a response to treatment.

The stromal compartment has drawn recent interest as a target for PDAC therapy. The stroma creates an inflammatory environment and promotes tumor progression; and the extent of activated stroma has been identified as a novel independent prognostic marker in PDAC[216]. Several potential PDAC therapies have targeted the stroma[217-219]. One method in particular aims at overcoming the immunosuppression often found in, and potentially caused by, cells of the stroma. CD40 agonists take advantage of the inflammatory cells found within the stroma[218].
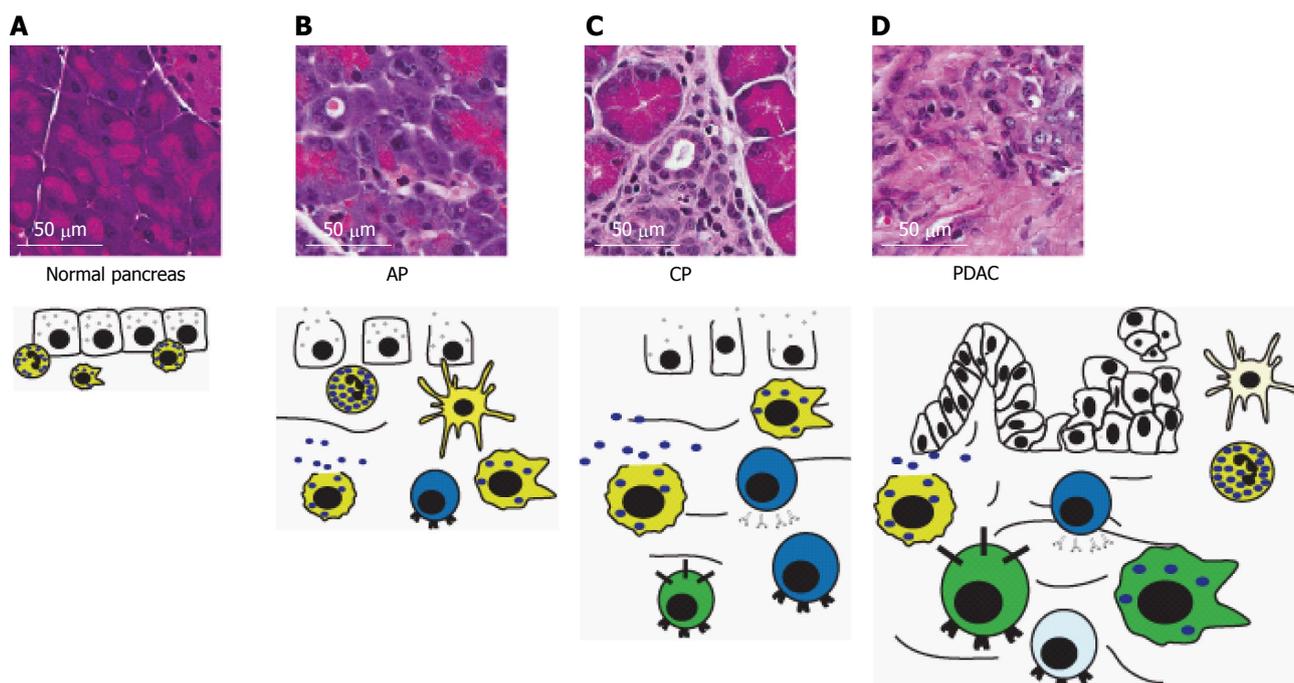
Figure 6 Immune cells in progressive pancreatic disease. A: The normal pancreas contains sparse, mostly innate, inflammatory cells and lacks the dense stroma typically seen in chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC); B: Acute pancreatitis (AP) is characterized by acinar degranulation, edema and recruitment of mostly innate inflammatory cells, but also some T-lymphocytes in response to acinar damage. Pancreatic mast cells begin to degranulate; C: CP is characterized by development of stroma surrounding degranulated acinar cells, acinar-to-ductal metaplasia and edema. Additionally, there is an increased presence of macrophages, and T- and B-lymphocytes, and further degranulation of mast cells; D: Development of pancreatic intraepithelial neoplasias and subsequent PDAC leads to a significant increase in immunosuppressive cell types including tumor-associated macrophages, myeloid-derived suppressor cells, and Tregs. Degranulated mast cells, neutrophils, dendritic cells and B- and T-lymphocytes are also present. However, T-lymphocytes and dendritic cells are typically inhibited and defective, respectively. Size of immune cell represents relative abundance. Cell color denotes immune cell type: yellow, innate immune cells; blue, adaptive immune cells; green, immunosuppressive cells. For description of graphical representation of cell types, see Figure 3 legend. Cells that are inactivated or defective are represented by a lighter color.

CD40 is the co-stimulatory factor needed for activation of the T-lymphocyte-dependent anti-tumor response of the immune system. It is thought that activating stromal T-lymphocytes may overcome the immunosuppressive environment that is a hallmark of PDAC. Co-treatment with a CD40 agonist and gemcitabine showed therapeutic efficacy in patients with metastatic PDA[218].

Whereas cancer immunotherapy has typically involved treatment with cancer antigens to stimulate or boost the anti-cancer immune response, the immunosuppressive environment found in PDAC presents obvious challenges. Vaccination with self-antigens has been associated with induction of immunosuppressive cell types, thus potentiating, rather than inhibiting, tumor growth[220]. Successfully overcoming the immunosuppressive environment that characterizes PDAC will likely require a multifaceted approach due to the multiple mechanisms by which tumor-associated immune dysfunction seems to occur. However, new evidence suggests that immunotherapy can be successful for pancreatic cancer, if stimulation of the immune system is combined with control over the immunosuppressive environment[221,222]. Developing new methods to overcome immunosuppression or exploit the immune response to target PDAC may be utilized in combination with conventional or novel chemotherapy to enhance the survival of this currently deadly disease.

CONCLUSION

The immune system protects the host from invading pathogens and foreign materials. The aberrant expression profile and uncontrollable proliferation that characterizes tumor cells should allow for recognition as non-self by the immune system. However, we now know that from a very early stage in PDAC development, the ability of the immune system to identify and eliminate neoplastic cells is compromised. This suggests that an immunosuppressive environment is established early in tumor development to effectively thwart the immune response to neoplastic cells at the onset of tumor development. Figure 6 depicts the progressive changes in immune cell infiltrate found during various stages of pancreatic disease.

Based on data reviewed here, neoplastic cells produce compounds (such as GM-CSF) at a very early stage of pancreatic cancer development, that recruit immunosuppressive immune cells, potentially facilitating progression to later PanIN stages and PDAC[190-192,194]. Once PDAC cells are present, they actively prevent the maturation of dendritic cells, inhibiting their antigen presenting activity and effectively cutting off a major communication between the innate and adaptive immune response[182]. In addition, immunosuppressive Tregs and MDSCs accumulate in the blood, stroma and PDAC tissue and inhibit T-lym-

phocyte proliferation[6,140,141,144-146]. In this setting, even immune cell types considered pro-inflammatory add to the tumor-supportive environment: neutrophils accumulate in the stroma and secrete proteases that aid in EMT, tumor motility and invasion[161,163] and mast cells accumulate in the tumor tissue and express factors used by the tumor to sustain its growth[152]. It is clear that a complex relationship exists between the immune system and the developing pancreatic cancer, and that these interactions have important implications for disease prevention and control. Immunotherapy can potentially be a powerful component of PDAC treatment. Further study of the mechanisms by which immunosuppression is initiated in PDAC, and ways to overcome it, will facilitate the development of this treatment option[22,104,223-249].

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Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection

Andrew E Becker, Yasmin G Hernandez, Harold Frucht, Aimee L Lucas

Andrew E Becker, Yasmin G Hernandez, Aimee L Lucas, Henry D. Janowitz Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States
Harold Frucht, Division of Digestive and Liver Diseases, Columbia University College, New York, NY 10032, United States
Author contributions: All authors were involved in the design of the manuscript; Becker AE wrote the manuscript; Lucas AL, Hernandez YG and Frucht H reviewed and revised the manuscript.
Correspondence to: Aimee L Lucas, MD, MS, Henry D. Janowitz Division of Gastroenterology, Icahn School of Medicine at Mount Sinai, One Gustave L Levy Place, Box 1069, New York, NY 10029, United States. aimee.lucas@mssm.edu
Telephone: +1-212-2410101 Fax: +1-646-5378647

Abstract

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States, with over 38000 deaths in 2013. The opportunity to detect pancreatic cancer while it is still curable is dependent on our ability to identify and screen high-risk populations before their symptoms arise. Risk factors for developing pancreatic cancer include multiple genetic syndromes as well as modifiable risk factors. Genetic conditions include hereditary breast and ovarian cancer syndrome, Lynch Syndrome, familial adenomatous polyposis, Peutz-Jeghers Syndrome, familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, cystic fibrosis, and ataxia-telangiectasia; having a genetic predisposition can raise the risk of developing pancreatic cancer up to 132-fold over the general population. Modifiable risk factors, which include tobacco exposure, alcohol use, chronic pancreatitis, diet, obesity, diabetes mellitus, as well as certain abdominal surgeries and infections, have also been shown to increase the risk of pancreatic cancer development. Several large-volume centers have initiated such screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published. The focus of this review will be both the genetic and modifiable risk factors implicated in pancreatic cancer, as well as a review of screening strategies and their diagnostic yields.

Core tip: Risk factors for developing pancreatic cancer include multiple genetic syndromes as well as modifiable risk factors. These factors can raise the risk of developing pancreatic cancer up to 132-fold over the general population. Several large-volume centers have initiated screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published. The focus of this review will be both the genetic and modifiable risk factors implicated in pancreatic cancer, as well as a review of screening strategies and their diagnostic yields.

INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States, with an estimated over 45000 diagnoses and 38000 deaths in 2013[1]. Pancreatic ductal adenocarcinomas (PDAC) arise from the exocrine pancreas and account for 95% of pancreatic cancers. The lifetime risk of developing pancreatic cancer

Table 1 Selected pancreatic ductal adenocarcinoma genetic risk factors			
Risk factor	**Gene**	**Increased PDAC risk**	**Other associated cancers**
Hereditary breast and ovarian cancer syndrome	*BRCA1, BRCA2, PALB2*	2-3.5	Breast, ovarian, prostate
Lynch syndrome (hereditary non-polyposis colorectal cancer)	*MLH1, MSH2, MSH6, PMS2, EPCAM*	8.6	Colon, endometrium, ovary, stomach, small intestine, urinary tract, brain, cutaneous sebaceous glands
Familial adenomatous polyposis	*APC*	4.5-6	Colon, desmoid, duodenum, thyroid, brain, ampullary, hepatoblastoma
Peutz-Jeghers syndrome	*STK11/LKB1*	132	Esophagus, stomach, small intestine, colon, lung, breast, uterus, ovary
Familial atypical multiple mole melanoma pancreatic carcinoma syndrome	*P16INK4A/CDKN2A*	47	Melanoma
Hereditary pancreatitis	*PRSS1, SPINK1*	69	
Cystic fibrosis	*CFTR*	3.5	
Ataxia-telangiectasia	*ATM*	Increased	Leukemia, lymphoma
Non-O blood group		1.3	
Familial pancreatic cancer	Unknown	9 (1 FDR) 32 (3 FDRs)	

PDAC: Pancreatic ductal adenocarcinomas; FDR: First-degree relative.

Table 2 Selected pancreatic ductal adenocarcinoma modifiable risk factors	
Risk factor	**Increased PDAC risk**
Current cigarette use	1.7-2.2
Current pipe or cigar use	1.5
> 3 alcoholic drinks per day	1.2-1.4
Chronic pancreatitis	13.3
BMI > 40 kg/m^2, male	1.5
BMI > 40 kg/m^2, female	2.8
Diabetes mellitus, type 1	2.0
Diabetes mellitus, type 2	1.8
Cholecystectomy	1.2
Gastrectomy	1.5
Helicobacter pylori infection	1.4

PDAC: Pancreatic ductal adenocarcinomas; BMI: Body mass index.

is 1.49%, or 1 in 67, with incidence increasing with age[2]. Epidemiologically, the incidence rates of PDAC are higher in males, African Americans, and lower socioeconomic status groups[1].

Both genetic and modifiable risk factors contribute to the development of PDAC. A hereditary component has been identified in approximately 10% of cases, with a specific germline mutation being implicated in 20% of those cases[3,4]. These genetic conditions, including the hereditary breast and ovarian cancer syndrome (HBOC), Lynch syndrome (HNPCC), familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS), familial atypical multiple mole melanoma syndrome (FAMMM), hereditary pancreatitis (HP), cystic fibrosis (CF), and ataxia-telangiectasia (AT), have been shown to raise the risk of PDAC anywhere from 2 to 132-fold (Table 1)[5-7]. Modifiable risk factors, which include tobacco exposure, alcohol use, chronic pancreatitis, diet, obesity, diabetes mellitus, as well as certain abdominal surgeries and infections have also been identified as increasing the risk of PDAC (Table 2).

PDAC is nearly universally lethal: less than 20% of patients are surgical candidates at the time of presenta-

tion, and the median survival for non-resected patients is 3.5 mo[8]. Even among those patients who are candidates to undergo pancreatectomy, the median survival is 12.6 mo[8]. However, by identifying and screening patients at an increased risk of developing PDAC, detection of precursor and early-stage lesions may allow diagnosis at a still surgically-resectable stage. Several large-volume centers have initiated screening protocols, and consensus-based guidelines for screening high-risk groups have recently been published[3,9]. The focus of this review will be both the genetic and non-genetic risk factors implicated in PDAC, as well as screening strategies and their diagnostic yields.

PDAC RISK FACTORS

PDAC risk factors: Genetic

It has been reported that up to 10% of PDAC have a hereditary component[4]. A 2009 meta-analysis demonstrated that having just one affected relative resulted in an 80% increased risk of developing PDAC[10]. Specific mutations in multiple genes have been implicated in causing roughly 10% of PDAC, with varying penetrance and degree of increased cancer risk for each mutation (Table 1)[11,12]. Identification and stratification of individuals at increased risk of having genetic mutations may allow for a group of patients that will benefit from early detection of these pancreatic neoplasms, as well as targeted, gene-specific therapy.

Hereditary breast and ovarian cancer syndrome and other fanconi anemia genes: *BRCA1*, *BRCA2/FANCD1*, *PALB2/FANCN*, *FANCC*, and *FANCG*: Fanconi anemia is an autosomal recessive disease characterized by multiple congenital anomalies, bone-marrow failure, and increased susceptibility to malignancy, including acute myeloid leukemia and head and neck squamous cell carcinoma[13,14]. There are 15 Fanconi anemia genes, and products of these genes are involved in multiple DNA repair

mechanisms, including the *BRCA1/2* pathway[13,14]. The incidence of the disease is 1 in 100000 live births, and the carrier rate of Fanconi anemia mutations is estimated at 1 in 300[13,15].

HBOC is characterized by early-onset breast and ovarian cancers resulting from monoallelic germline mutations in the *BRCA1* or *BRCA2* (also known as *FANCD1*) genes. These tumor suppressor genes code for proteins that repair double-stranded DNA breaks. While *BRCA2* codes for a Fanconi anemia protein, the *BRCA1* protein directly interacts with the FANCA protein[16]. *BRCA1/2* mutations have been shown to have a population frequency of 1.0%, with a higher concentration within the Ashkenazi Jewish population (2.3%)[17,18]. These genes have high penetrance with respect to female breast cancer (cumulative risk by age 70 of 57% for *BRCA1* and 49% for *BRCA2*) and ovarian cancer (cumulative risk by age 70 of 40% for *BRCA1* and 18% for *BRCA2*), and lower rates for male breast cancer (cumulative risk by age 70 of 1.2% for *BRCA1* and 6.8% for *BRCA2*) as well as PDAC[19]. While a few large studies have indicated that *BRCA1* mutations are associated with a roughly 2-fold increased risk of PDAC, the mutation is rarely seen in PDAC families without a strong history of breast cancer[6,7,20]. Additionally, not all studies have found an increased risk of PDAC among the *BRCA1* cohort[21]. On the other hand, the evidence for an association between *BRCA2* germline mutations and PDAC is more clearly defined. With a relative risk of at least 3.5, *BRCA2* mutations have been identified as the most common known inherited cause of PDAC: studies have found deleterious mutations in the *BRCA2* gene in 17%-19% of familial pancreatic cancer families and 7.3% of apparently sporadic pancreatic cancers[22-25]. Our group has demonstrated an increased prevalence of *BRCA1* mutations (8.3%) and *BRCA2* mutations (10.8%) in a cohort of unselected Ashkenazi Jewish patients who underwent surgical resection for PDAC and IPMN; half of those *BRCA1/2*-associated tumors demonstrated loss of heterozygocity[26]. In a registry study of *BRCA1* and *BRCA2* families, there was a significantly earlier age of onset (age 63 for each) for PDAC, compared to that found in the SEER database (age 70)[27].

PALB2, or partner and localizer of *BRCA2* (also known as *FANCN*), is a gene that codes for a protein which stabilizes the *BRCA2* protein as it repairs DNA. *PALB2* is known to be a breast cancer susceptibility gene and has been found to be mutated in up to 3% of familial PDAC[28,29]. While some large registry cohort studies have not found *PALB2* mutations to increase the relative risk of PDAC, other groups have identified *PALB2* mutations in multiple familial pancreatic cancer families[30-33]. Additionally, it has been demonstrated that relatives of *PALB2* mutation carriers have a 6-fold increased risk of PDAC compared to relatives of those with the wild-type gene[34].

Mutations in two other Fanconi anemia proteins, specifically *FANCC* and *FANCG,* have shown loss of heterozygosity in young-onset (< 55 years of age) PDAC[35,36]. No studies to date have found an increased risk of PDAC associated with mutations in these genes.

Targeted therapy is a promising area of research for genes in this pathway. Cells deficient in *BRCA1, BRCA2/ FANCD1, PALB2/FANCN, FANCC* or *FANCG* must use DNA repair mechanisms that are more error prone and resultant mutations are more likely to result in cell death. Thus, agents that induce DNA damage or inhibit other repair mechanisms may affect deficient cells more than fully-functional cells[37]. *In vitro* cells deficient in these proteins and *in vivo* cells in mice were shown to be hypersensitive to alkylating agents such as mitomycin C, cisplatin, chlorambucil, and melphalan, whereas normal cells were unaffected[38,39]. Additionally, poly (ADP-ribose) polymerase (PARP) inhibitors have been shown to have anti-tumor activity in multiple other human cancers[40]. There have been case reports of complete pathological response of *BRCA2*-associated PDAC to PARP inhibitors, and clinical trials are currently underway[41].

Lynch syndrome (or HNPCC): *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*: Lynch syndrome, the most common inherited colorectal cancer syndrome, is characterized by early-onset colorectal cancer as well as a predisposition to cancer of the endometrium, ovary, stomach, small intestine, urinary tract, brain, pancreas and cutaneous sebaceous glands[42]. The incidence of this syndrome has been postulated to be between about 1:660 to 1:2000[43]. The *MSH2*, *MSH6*, *MLH1*, *PMS2*, and *EPCAM* genes, which are mutated in this syndrome, normally code for proteins involved in the DNA mismatch repair pathway which bind to mismatched double-stranded DNA and microsatellites to target and prepare them for repair[42]. Patients with Lynch syndrome have an 8.6-fold increased risk of developing PDAC compared to the general population[44]. These pancreatic tumors often have a characteristically medullary appearance, with prominent lymphocytic infiltration and microsatellite instability[44,45].

FAP: *APC*: FAP is characterized by the early development of hundreds to thousands of colorectal adenomatous polyps; some of these polyps inevitably progress to malignancy, conferring an almost 100% risk of colorectal cancer by age 40[46]. There is also an increased risk of extracolonic cancers including desmoid, duodenum, thyroid, brain, ampullary, pancreas, and hepatoblastoma tumors[47]. The incidence of FAP in the Northern European population is 1 in 13000-18000 live births in the Northern European population[48,49]. FAP is caused by a mutation in the *APC* gene, a tumor suppressor gene which codes for a scaffolding protein responsible for targeting β-catenin for destruction, as well as acting as a control on progression of the cell cycle and a microtubule stabilizer[47]. Specifically, the relative risk of PDAC in FAP is reported to be 4.5 to 6-fold, although it is uncertain if this represents a true increased risk of PDAC or reflects misclassification

of ampullary carcinomas[50,51]. There also exists a subset of the FAP population with an attenuated phenotype, known as attenuated FAP (AFAP) that is also caused by a mutation in the *APC* gene; this population has fewer colorectal adenomatous polyps (10-100) and a fifteen-year delay in the onset of colorectal cancer compared to those with FAP[52]. Compared to FAP, AFAP is associated with a lower risk of extracolonic cancers[53].

PJS: *STK11/LKB1*: PJS is characterized by hamartomatous gastrointestinal polyposis and distinctive mucocutaneous pigmentation found most commonly on the lips or perioral region[45,54]. PJS, with an estimated frequency of 1:8300 to 1:280000, is associated with an inherited mutation in the *STK11/LKB1* gene, a tumor suppressor gene which encodes for a serine/threonine kinase[45]. While the exact mechanism by which the *LKB1* gene acts as a tumor suppressor is unknown, PJS tumors have shown less activated AMP-kinase, which results in mammalian target of rapamycin hyperactivation[55]. Additionally, *LKB1* haploinsufficiency has been shown to cooperate with *K-ras* to cause PDAC in the mouse model, through a decrease in growth arrest[56]. A 2000 meta-analysis demonstrated that PJS is associated with a relative risk of 15.2 for all cancers and a 93% overall rate of cancer by age 64[5]. The study found a statistically significant increased risk of esophageal, stomach, small intestine, colon, pancreas, lung, breast, uterus, and ovarian cancers, including a relative risk of 132 for PDAC.

FAMMM: *p16INK4A/CDKN2A*: FAMMM is characterized by malignant melanoma in one or more first-degree relatives (FDRs) or second-degree relatives (SDRs) and multiple, atypical melanocystic nevi[55]. The prevalence of FAMMM is unknown. While there is variability in the underlying genetics of this syndrome, a germline mutation in the *p16INK4A* (also known as *CDKN2A* or *MTS1*) gene has been found in approximately 38% of the cases of this syndrome[57,58]. FAMMM with this particular mutation, which confers a 60%-90% risk of melanoma by age 80, is called FAMMM pancreatic carcinoma syndrome (FAMMM-PC) because those with the *p16INK4A* mutation have also demonstrated an increased risk of PDAC[59-62]. This gene, which codes for the *p16* protein, is a tumor suppressor gene involved in the regulation of cell cycle progression. A study following 19 FAMMM families over seventy years found a 13 to 22-fold increased risk of developing PDAC in those with this *p16INK4A* mutation; conversely, they found no cases of PDAC in those without this mutation[63]. More recently, a relative risk of PDAC of 47 was demonstrated among those with this *p16INK4A* mutation compared to the general population[64]. The risk of PDAC was even more apparent when looking at those under 55 years of age: a Swedish study found the relative risk to be 65-fold for *p16* mutation carriers[61].

HP and CF: *PRSS1*, *SPINK1* and *CFTR*: HP is char-

acterized by recurrent attacks of acute pancreatitis starting in childhood, which can lead to pancreatic failure[65]. About 80% of HP is caused by a germline mutation in the *PRSS1* gene, which codes for the prodigestive enzyme trypsinogen[66]. Defective mutations result in either premature activation or reduced deactivation of the enzyme, leading to pancreatic injury. The *SPINK1* gene codes for a serine protease inhibitor that inhibits active trypsin; mutations in this gene have also been associated with various forms of pancreatic disease, including pancreatitis[67]. HP has an 80% penetrance rate[68]. A 2010 meta-analysis found a relative risk of 69 for PDAC for patients with HP compared to the general population[69].

Additionally, homozygous mutations in the autosomal recessive *CFTR* gene cause cystic fibrosis, which is associated with both a younger age of onset (median age of 35 years) and 5.3-fold greater risk of the development of PDAC[70]. However, even when a *CFTR* gene mutation is inherited in a heterozygous fashion, it has been demonstrated that this confers a 4-fold greater chance of developing chronic pancreatitis[66,71,72].

The presence of chronic inflammation in pancreatitis is thought to be the primary mechanism by which PDAC develops. A few mechanisms have been suggested as methods by which inflammation leads to PDAC[73]. Inflammatory cytokines such as IL-6 and IL-11 may induce the proliferation and facilitate survival of malignant and premalignant cells through the activation of multiple transcription factors, including STAT3 and NF-κB. Additionally, chronic inflammation may suppress immunosurveillance as well as inhibit oncogene-induced senescence, which would allow the lesion to develop unchecked. It has been suggested that increased activation of pancreatic stellate cells leads to fibrosis *via* increased cell proliferation and inflammation[74].

AT: *ATM*: AT is an autosomal recessive, progressive neurologic disorder characterized by early ataxia and later telangiectasias of the blood vessels on exposed areas of the skin and eyes, with cerebellar ataxia, varied immune dysfunction, an extreme sensitivity to ionizing radiation, and an increased risk of cancers, particularly leukemias and lymphomas[75-77]. The estimated incidence of AT is 1 in 40000-300000 live births, and the disease is caused by a homozygous mutation in the *ATM* gene, which codes for a serine/threonine kinase involved in DNA repair[77]. Monoallelic A*TM* mutation carrier status, an estimated 1.4% of the United States population, is also associated with an increased risk of cancer, especially that of the female breast[78,79]. Among the families of those with AT, the rate of PDAC is at least twice that of the general population[80,81]. A 2012 study of a familial pancreatic cancer cohort found monoallelic ATM mutations in 2.4% of the PDAC probands, and that number increased to 4.6% of the patients with at least 3 FDRs with PDAC. Loss of heterozygosity of the *ATM* gene was found in the only patient with available tumor tissue in the study[77].

Non-O blood group: Non-O blood groups have also been associated with a higher risk of PDAC[82-84]. Multiple prospective and case-control studies across different countries as well as a genome-wide association study demonstrated an increased risk of PDAC among those with non-O blood groups; additionally, a 2010 meta-analysis found that having an O blood group was associated with a relative risk of 0.79 for the development of PDAC[83,85]. In fact, it was demonstrated that each additional non-O allele conferred a larger risk of PDAC[86]. Interestingly, it was shown that the association between non-O blood groups and PDAC was largest in individuals colonized by CagA-negative *Helicobacter pylori* (*H. pylori*)[84]. While it has been postulated that the increased cancer risk is related to a chronic host inflammatory state, it has been found in one study that non-O blood groups do not increase the risk of chronic pancreatitis[83,87].

FPC: Unknown gene: Familial pancreatic cancer (FPC), defined as having 2 or more FDRs with PDAC with no known genetic cause, is responsible for up to roughly 80% of clustering PDAC[3]. The National Familial Pancreas Tumor Registry at Johns Hopkins demonstrated a nine-fold greater risk of developing PDAC among individuals with an FDR with PDAC in the setting of FPC, compared to a 1.8-fold greater risk for those with an FDR with sporadic PDAC[12]. Additionally, among FPC kindreds, having two or three FDRs with PDAC was associated with a 6.4-fold and 32-fold greater risk of developing PDAC, respectively.

Additionally, studies of the European Registry of Hereditary Pancreatitis and FPC as well as the German National Case Collection for FPC Registries have described anticipation (developing PDAC roughly 10 years earlier than their affected parent) in 59%-80% of over 100 FPC families[33,88]. Finally, segregation analyses have shown evidence for a yet-unidentified autosomal dominant, high-risk allele influencing the onset age of PDAC present in 7/1000 individuals[89]. The *palladin* gene, a proto-oncogene overexpressed in some sporadic pancreatic tumors has also been found to be mutated in affected members of one PDAC family[90-92]. This gene codes for a cytoskeleton protein that promotes tumor invasion in fibroblasts[90].

PDAC risk factors: Modifiable

Multiple modifiable risk factors are associated with an increased risk of developing PDAC (Table 2). Since PDAC has such a low incidence rate and most of the associated relative risks (with the exception of chronic pancreatitis) are low, greater improvements in PDAC morbidity and mortality may be possible with lifestyle modification.

Tobacco use: Smoking is the largest identifiable and modifiable risk factor for PDAC, contributing to 20%-35% of PDAC cases[93-95]. A 2008 meta-analysis of 82 studies demonstrated an increased risk of PDAC development for both current cigarette (relative risk of 1.74) and pipe

or cigar (1.47) users[93]. A 2012 pooled analysis found the risk of current cigarette use to be 2.2-fold[96]. Additionally, both studies found increased smoking intensity and cumulative smoking dose to increase the risk for development of PDAC. Even after 10 years of smoking cessation, a modestly elevated relative risk of 1.48 remains[93]. However, multiple studies have demonstrated a risk of PDAC among former smokers to be similar to non-smokers after up to 15-20 years of cessation[96-100]. Finally, exposure to second-hand tobacco smoke has been found to increase the risk of PDAC by 21%[101].

It is likely that PDAC develops from exposure to tobacco-related carcinogens through circulating blood, especially given a similar rate of tobacco-related neoplasm in the kidney and stomach[93]. These carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons, as well as their metabolites, cause mutations in both protoncogenes (K-ras) and tumor suppressors (p53)[102,103]. Tobacco smoke also directly contributes to pancreatic inflammation[103].

Smoking is particularly harmful in certain cohorts. For patients with HP, smoking has been demonstrated to more than double the risk of PDAC and lower the age of cancer onset by 20 years[95]. For members of FPC families, one study found cigarette smoking resulted in a 4-fold increased risk over non-smokers, as well as lowering the age of onset of PDAC by 10 years[104]. Another study demonstrated an incidence ratio of 19.2 for members of PDAC families who had ever smoked cigarettes *vs* 6.25 for those who had never smoked at all[12].

Alcohol use: While alcohol has been found to be associated with PDAC, the current evidence indicates that it is limited to heavy alcohol usage only: pooled data and meta-analyses have found three or more drinks per day to be associated with a 1.22 to 1.36-fold increased risk of developing PDAC, with a dose-response relationship[105,106]. It is known that heavy alcohol usage does contribute to pancreatitis, which may be a method by which it increases the risk of PDAC[107]. Additionally, metabolites of alcohol, including acetaldehyde (a carcinogen) and fatty acid ethyl esters, as well as ethanol itself (a carcinogen) can cause pancreatic inflammation as well as directly contribute to carcinogenesis[103].

Chronic pancreatitis: A 2010 meta-analysis demonstrated a relative risk of 13.3 for developing PDAC in those with chronic pancreatitis, with a ten to twenty year lag between the incidences of pancreatitis and pancreatic malignancy[69]. As with hereditary pancreatitis, chronic inflammation seen in chronic pancreatitis is thought to be the mechanism by which PDAC develops. Far and away, the most common cause of chronic pancreatitis is alcohol abuse, which is responsible for 60%-90% of cases[108]. As with HP, chronic inflammation is thought to be the mechanism by which PDAC develops in chronic pancreatitis. Inflammatory cytokines may induce cellular proliferation, as well as reduce immunosurveillance and inhibit

senescence, allowing the lesion to continue to grow[73].

Diet and obesity: Meta-analyses have demonstrated an increased risk of PDAC associated with a diet including red meat in men (relative risk of 1.29), and processed meat in both men and women (1.19)[109]. Another meta-analysis found that there was a relative risk of 1.12 for developing PDAC for each $5kg/m^2$ increase in body mass index (BMI)[110]. A large 2003 study found a BMI of over 40 to be associated with a relative risk of PDAC of 1.49 for men and 2.76 for women[111]. Interestingly, a 2009 study found being overweight or obese at a younger age to be associated with a younger age of onset of PDAC; the study also found those who had a BMI over 25 from ages of 30 to 79 had reduced PDAC survival[112]. The method by which fat consumption may lead to PDAC includes pancreatic hypertrophy and hyperplasia in response to cholecystokinin-mediated lipase secretion from the presence of fat in the duodenum, which puts the pancreatic exocrine glands at an increased risk of carcinogenesis[102]. Additionally, hyperglycemia, abnormal glucose levels, and insulin resistance are all associated with an increased risk of PDAC[112-117].

Diabetes mellitus: type 1, type 2, type 3c: Meta-analyses have demonstrated associations between both type 1 and type 2 diabetes mellitus (DM) and pancreatic cancer, with odds ratios of approximately 2.0 and 1.8, respectively[109,118,119]. Twenty-five to 50% of patients with PDAC will have developed DM 1-3 years prior to their PDAC diagnosis; however, the relative risk of pancreatic cancer drops as time from type 2 DM diagnosis increases, indicating that DM may in fact be an early manifestation of the cancer[118,120,121]. Also, while new-onset DM is not specific for PDAC (less than 1% of adult-onset DM patients will develop PDAC within 3 years), large cohort studies in the United States and Sweden have demonstrated differing relative risks for those with a long history of DM *vs* those with new-onset DM: having DM for a longer time is associated with a decreased PDAC risk compared to newly-diagnosed DM[121-124]. In addition, associated new-onset DM has been shown to resolve after tumor resection[114,125,126].

A different diabetes diagnosis, type 3c (pancreatogenic) DM, or diabetes associated with acute or chronic disease of the pancreas, which is up to 8% of all diabetes, may confer an even higher risk of pancreatic cancer, especially in those patients with chronic pancreatitis[121,127-129]. Type 3c DM occurs in up to 30% of patients with PDAC and is associated with deficiencies in islet hormones such as insulin, glucagon, and pancreatic polypeptide[121]. Most frequently, the insulin resistance is actually hepatic resistance, with relatively normal peripheral insulin sensitivity; this is thought to be a result of a deficiency of pancreatic polypeptide, which has been shown to affect hepatic insulin receptors[128,130]. In patients with pancreatic polypeptide deficiency, this hepatic insulin resistance has been shown to return to normal with the replacement of the hormone[128,131,132].

Insulin is growth promoting, and thus chronic insulinemia may result in increased cellular proliferation and decreased apoptosis, a mechanism by which PDAC may eventually develop[110,112,117]. This is mediated through both increased levels of insulin, as well as insulin-like growth factor-1, which also results from hyper-insulinemia[102]. Additionally, the oxidative stress from hyperglycemia may be the cause of cell damage that could lead to the development of neoplasm.

DM treatment choice has been demonstrated to modulate pancreatic risk. One case-control study found a relative risk of 2.89 for pancreatic cancer in those with DM; this risk decreased to 2.12 with treatment by oral hypoglycemic agents and increased to 6.49 by treatment with insulin[98]. This is consistent with evidence that insulin can promote pancreatic cancer cell proliferation[133]. In particular, treatment with metformin has been shown to decrease overall cancer risk in diabetic patients[134,135]. Multiple studies have demonstrated a decreased risk of pancreatic cancer among diabetics treated with metformin[135-137]. Specifically, one study demonstrated that treatment with metformin conferred a relative risk of pancreatic cancer of 0.30, *vs* 2.78 with treatment with insulin[135].

Surgery and infection: A meta-analysis found a relative risk of PDAC of 1.23 for those with a history of a cholecystectomy[138]. The mechanisms suggested by which cholecystectomy increases the risk of PDAC include increased cholecystokinin levels, which have been shown to stimulate the growth of human pancreatic cancer cell lines and promote pancreatic carcinogenesis in hamsters, as well as increased degradation of bile salts to secondary bile acids, which have a pancreatic carcinogenic effect in hamsters[138-142].

Another meta-analysis has demonstrated a relative risk of 1.54 for developing PDAC post-gastrectomy, with a higher risk found for Billroth II resections than Billroth I resections[143,144]. The reasons postulated for higher rates of pancreatic carcinogenesis include a post-gastrectomy environment favorable for bacteria that increase levels of DNA-damaging N-nitrosamine carcinogens, increased rates of *H. pylori* seropositivity, and increased rates of recurrent acute pancreatitis in Billroth II resections[144].

Evidence suggests *H. pylori* infection is associated with PDAC: a 2011 meta-analysis found an increased odds ratio of 1.38[145]. The definitive method by which *H. pylori* infection contributes to the development of PDAC is unknown, but may be related to the inflammatory mediators and angiogenic factor secretion associated with chronic infection[145]. There is some evidence for a link between hepatitis B infection and pancreatic cancer, as well as possibly hepatitis C; however, the method by which these infections contribute to PDAC is unknown[146,147].

Hydrocarbon exposure: While studies have shown correlations between pancreatic cancer and various expo-

sures, the most consistent exposures linked to development of pancreatic neoplasm are chlorinated hydrocarbons and polycyclic aromatic hydrocarbons[148]. However, it is important to note that consistently statistically significant results have not been found with either of these two occupational exposures.

PDAC STAGING, RISK STRATIFICATION AND SCREENING

Staging, prognosis, and the case for screening

The five-year PDAC survival rate of 6% is dismal, largely because the majority of patients are diagnosed at an advanced stage[1]. Surgical resection is the only curative treatment for pancreatic cancer. However, only pre-cancerous or early-stage (Ⅰ-Ⅱ) PDAC is surgically resectable. Since five-year survival rate for patients diagnosed with Stage ⅠA disease is 19 times that of those diagnosed with Stage Ⅳ disease (13.6% *vs* 0.7%), greater improvements in survival may be seen if we focus on shifting the diagnosis of PDAC from a late stage to an early or pre-cancerous stage[8]. Unfortunately, early-stage PDAC is usually clinically silent, highlighting the need for improved methods of early detection of precursor and early stage lesions. This provides the rationale for screening programs to detect precursor and early stage lesions.

PDAC precursors

World Health Organization guidelines suggest that in order to screen for a cancer, there must be a recognizable latent or early stage of the disease that can be tested for and managed effectively[148]. Several pancreatic lesions meet the criteria for a precursor to PDAC: pancreatic intraepithelial neoplasms (PanINs), mucinous cystic neoplasms (MCNs), and intraductal mucinous cystic neoplasm (IPMNs)[149,150].

PanIN: PanINs are non-invasive, non-mucin-producing, small epithelial neoplasms[150,151]. There are 3 grades of PanINs, classified by degree of atypia: PanIN-1, PanIN-2, and PanIN-3. A 2003 study found PanIN lesions in 82% of pancreata with invasive cancer compared to just 28% of normal pancreata, as well as an increased number of high-grade PanIN lesions compared to low-grade PanIN lesions[152]. Multiple studies have found PanIN-3 lesions only in pancreata harboring other malignancies[152-154]. For PanIN lesions, there are three broad subsets of germline or somatic mutations that are usually found in concert in a pancreatic malignancy: (1) activation of oncogenes (*K-Ras, HER2*); (2) inactivation of tumor suppressor genes (*TP53, p16/CDKN2A, SMAD4/DPC4, BRCA1, BRCA2*); and (3) inactivation of genome maintenance genes (*MLH1, MSH2*)[151,155,156]. While PanINs are not visible on cross-sectional imaging, a 2006 study suggests that endoscopic ultrasound (EUS) may be able to detect lobular parenchymal atrophy associated with PanINs, particularly multifocal PanIN, and IPMNs[157].

Pancreatic cystic neoplasms: MCN and IPMN: Autopsies indicate that the prevalence of patients with a pancreatic lesion at death is about 24%; studies have found that magnetic resonance imaging (MRI) picks up incidental pancreatic cysts in patients with no pancreatic history in up to 13.5% of patients, and computed tomography (CT) in 2.6%[158-160]. The ability to detect precursor lesions before they invade and progress to pancreatic cancer is of the utmost importance. MCNs are cystic, mucin-producing epithelial neoplasms with ovarian-type stroma, detectable on cross-sectional imaging[150]. MCNs are much more common in females than males (95% female), and a significant percentage of the stroma cells stain positive for estrogen or progesterone receptors[161,162]. With a mean age of diagnosis of 45-50, MCNs usually arise in the body or tail of the pancreas (> 90%) and do not communicate with the larger pancreatic ducts[161-165]. Compared to non-invasive MCNs, malignant MCNs are diagnosed in older patients and are significantly larger, indicating that they most likely grow slowly over time[163,166]. The five-year survival rate for margin-negative, surgically resected non-invasive MCNs is close to 100%, but roughly 50% for invasive MCNs; however, their low frequency of invasion (12%) highlights the need for better characterization of tumor progression[161-163,166].

IPMNs, which include branch duct (BD-IPMN), main duct (MD-IPMN), and mixed types, are mucin-producing epithelial neoplasms that are also detected by cross-sectional imaging[167]. They are more common in the head of the pancreas, affect men more than women and have a mean age of diagnosis of about 65 years of age[166,168]. While BD-IPMNs and MD-IPMNs have the same age of presentation, BD-IPMNs are more common and frequently multifocal (21%-41% of cases) and less likely to progress to malignancy (11%-17% *vs* 44%-48% *vs* 45% for mixed IPMNs)[166,169-173]. Patients with resected BD-IPMNs also have a higher five-year survival rate (91%) than both MD-IPMNs (65%) and mixed IPMNs (77%)[166].

Patients with both MCNs and IPMNs have improved survival when lesions are resected before developing an invasive component: a study of 851 consecutive resected patients at Massachusetts General Hospital showed a five-year survival rate of 87% for those with invasive and non-invasive cystic lesions and just 62% in those with malignancy[172].

While it is important to continue to better our ability to identify these PDAC precursor lesions, this must be matched by an improvement in the capacity to accurately predict which of those lesions will progress to malignancies. Characterizing how these precursor lesions develop will help better guide future screening and subsequent treatment.

Screening modalities: Imaging and biomarkers

Imaging: EUS and MRI have demonstrated the most accuracy as screening modalities for PDAC in terms of detecting small, cystic lesions, while magnetic resonance

Table 3 Pancreatic ductal adenocarcinomas screening efforts and diagnostic yields *n* (%)

Ref.	Number screened	High-risk group	Initial imaging (if abnormal screening)	Diagnostic yield	Definition of diagnostic yield
Brentnall *et al*[182]	14	FPC	EUS + ERCP + CT	7 (50)	Dysplasia
Rulyak *et al*[183]	35	FPC	If symptomatic: EUS + ERCP	12 (34.3)	Dysplasia
			If asymptomatic: EUS (ERCP)		
Kimmey *et al*[184]	46	FPC	EUS (ERCP)	12 (26)	Dysplasia
Canto *et al*[185]	38	FPC, PJS	EUS (CT, ERCP, EUS-FNA)	2 (5.3)	PDAC, IPMN
Canto *et al*[186]	78	FPC, PJS	EUS + CT (ERCP, EUS-FNA)	8 (10.3)	IPMN, PanIN1-2
Poley *et al*[187]	44	FPC, BRCA, PJS, FAMMM, p53, HP	EUS (CT, MRI)	10 (23)	PDAC, IPMN on imaging
Langer *et al*[188]	76	FPC, BRCA, FAMMM	EUS + MRCP (EUS)	1 (1.3)	IPMN
Verna *et al*[181]	51	FPC, PJS, FAMMM, BRCA, HP, HNPCC	EUS and/or MRCP (EUS-FNA, ERCP)	6 (12)[1]	PDAC, IPMN, multifocal PanIN2-3
Ludwig *et al*[189]	109	FPC, BRCA	MRCP (EUS)	9 (8.3)	PDAC, IPMN, PanIN2-3, SCA on imaging
Vasen *et al*[190]	79	p16	MRI/MRCP, EUS if unable	7 (8.9)	PDAC
Al-Sukhni *et al*[191]	262	FPC, FDR of double-primary cancer, BRCA, PJS, HP, p16	MRI (ERCP, EUS, CT)	3 (1.1)[2]	PDAC
Schneider *et al*[33]	72	FPC, BRCA, PALB2, p16	EUS + MRCP (EUS)	4 (5.5)	MD-IMPN, multifocal PanIN23
				9 (12.5)	MD-IMPN, multifocal PanIN2-3, BD-IPMN
Canto *et al*[174]	216	FPC, BRCA, PJS	CT + MRI/MRCP + EUS (ERCP)	92 (42.6)	Pancreatic lesion

[1]Only 41 patients had imaging, resulting in yield of 14.6% (6/41); [2]Only 175 patients had imaging, resulting in yield of 1.7% (3/175). PDAC: Pancreatic ductal adenocarcinomas; HNPCC: Lynch syndrome; FAP: Familial adenomatous polyposis; PJS: Peutz-Jeghers syndrome; FAMMM: Familial atypical multiple mole melanoma syndrome; HP: Hereditary pancreatitis; FPC: Familial pancreatic cancer; endoscopic retrograde MRI: Magnetic resonance imaging; CT: Computed tomography; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; MCN: Mucinous cystic neoplasms ; IPMN: Intraductal mucinous cystic neoplasm; FNA: Fine needle aspirate.

cholangiopancreatography (MRCP) provides the best visualization of possible communication with the main pancreatic duct[9,174]. CT subjects patients to radiation and has a suboptimal detection rate compared to EUS and MRI. Abdominal ultrasound and endoscopic retrograde cholangiopancreatography are not used as screening modalities for PDAC[9].

Biomarkers: Due to high cost, relative inability of non-invasive imaging modalities to detect small and solid tumors, and the modest risks associated with screening techniques like EUS, the use of biomarkers for the early detection of PDAC is an important frontier[175].

Carbohydrate antigen 19-9 (CA 19-9) is the only FDA approved blood biomarker test for PDAC[176]. However, due to the low prevalence of PDAC in the population, CA 19-9 is recognized as a poor screening tool: a screening of over 10000 patients found only 4 cases of PDAC based on CA 19-9 levels; additionally, 3 of those cases were not resectable at diagnosis[176]. The sensitivity (70%), specificity (87%), positive predictive value (59%), and negative predictive value (92%) are still not high enough to be used regularly in healthy patients[176,177]. CA 19-9 levels do appear to be informative as a predictor of disease recurrence post-resection[176,178].

The literature surrounding pancreatic cancer biomarkers is vast: a 2009 analysis found over 2500 genes overexpressed at the mRNA or protein level[179]. There is ongoing research that suggests a future for gene expression profiling, proteomics, metabolomics, and microRNA

as diagnostic PDAC biomarkers.

Current screening guidelines

The low absolute risk of developing PDAC precludes population-wide screening at the current time, both from a cost-benefit and absolute harm perspective. Assuming a lifetime risk of developing PDAC of 1.49%, a hypothetical screening test with 90% sensitivity and specificity would have a positive predictive value (PPV) of just 12%, meaning that almost nine in ten positive screening results would be incorrect, with those patients subject to unnecessary stress and further testing[3]. Even a screening test with 95% sensitivity and specificity would result in a PPV of just 22%. Notwithstanding, the identification of genetic and environmental risk factors may provide opportunities to enrich the screening population with high-risk cohorts, which would drastically increase the PPV of screening results, with the hopes of identifying precursor or early-stage lesions in some high-risk individuals before the lesions progress to inoperable pancreatic cancer.

Brand *et al*[180] published recommendations for PDAC screening in 2007. They suggested that potential candidates for screening included: (1) *BRCA1, BRCA2, p16* mutation carriers with at least one FDR or SDR with PDAC; (2) a PJS family member (preferably confirmed germline mutation carrier); (3) HP patients; (4) a patient with 2 relatives in same lineage with PDAC, at least one of whom is an FDR of the patient; and (5) patients with ≥ 3 FDR, SDR or third-degree relatives with PDAC. They suggested that screening of these individuals

Table 4 Selected highlights

Selected recent advances	**Genetic risk factors**
	In 2009, the use of gene sequencing identified PALB2, which had previously been implicated in breast cancer, as a susceptibility gene for PDAC[28]
	Expression of the palladin gene has been shown to be upregulated by cohabitance of normal fibroblasts with epithelial cells expressing the K-Ras oncogene. In 2012, it was shown that the palladin gene, which codes for a cytoskeletal protein, promotes mechanisms for metastasis and outgrowth of tumerogenic cells[90]
	Also in 2012, gene sequencing indicated that ATM mutations result in a predisposition to PDAC; LOH was demonstrated in 2 kindreds with PDAC[77]
	Therapy
	For patients with diabetes, treatment with metformin is associated with a lower relative risk of pancreatic cancer[127,136,137]
	A 2011 case report detailing a complete pathological response of a BRCA2-associated pancreatic tumor to gemcitabine plus iniparib showed the potential for PARP inhibitors in the treatment of BRCA2-associated pancreatic cancer[41]. Similar clinical trials are currently underway
Screening	**Screening goals**
	The goal of PDAC screening is the detection and treatment of (1) resectable PDAC; (2) PanIN-3 lesions; and (3) IPMN with high-grade dysplasia
	Low prevalence and high risk cohort enrichment
	The low absolute risk of PDAC development precludes population-wide screening from a cost-benefit and absolute harm perspective. The opportunity to screen high-risk cohorts will vastly increase the PPV of a screening test
	Screening efforts
	Past screening efforts, using patients cohorts at a high risk of developing PDAC, have demonstrated diagnostic yields from 1.1% to 50%, depending on their definition of yield (Table 3). Current screening modalities may be costly and invasive, and therefore associated with some patient risk. Furthermore, the long-term implications for detection of small and clinically insignificant lesions are uncertain. Further studies are needed to determine appropriate surveillance
Anticipated future advances and screening possibilities	**Risk stratification**
	Personal, family, genetic and environmental history will allow risk stratification and development of tailored screening and surveillance programs
	Biomarkers
	Ongoing research that suggests a future for gene expression profiling, proteomics, metabolomics, and microRNA as diagnostic PDAC biomarkers
	Targeted therapy
	As with BRCA2-associated tumors and PARP inhibitors, tumor biology will increasingly dictate the subsequent therapy

PDAC: Pancreatic ductal adenocarcinomas; IPMN: Intraductal mucinous cystic neoplasm.

should occur only under research protocol conditions, and required a threshold of at least 10-fold increased risk of PDAC. However, there was no consensus on the approach to screening, when to begin screening, and frequency of surveillance.

In 2011, the International Cancer of the Pancreas Screening (CAPS) Consortium held a conference with a panel of 49 experts from multiple disciplines, with the goal "to develop consortium statements on screening, surveillance and management of high-risk individuals with an inherited predisposition to PC [pancreatic cancer]"[9]. There was agreement that detecting and treating invasive resectable PDAC as well as multifocal PanIN-3 and IPMN with high-grade dysplasia should be considered a successful outcome of a screening or surveillance program.

The CAPS consortium suggested guidelines for PDAC screening, based on evidence of increased PDAC risk[9]. The statements agreed upon (> 75% consensus) were to screen candidates with: (1) two FDRs with PDAC; (2) two blood relatives with PDAC and at least one FDR; (3) PJS; (4) *BRCA2* mutation carriers with either one FDR with PDAC or at least two affected family members; (5) *PALB2* mutation carriers with at least one FDR with

PDAC; (6) *p16* mutation carriers (FAMMM) with at least one FDR with PDAC; and (7) lynch syndrome and one FDR with PDAC. While they agreed that initial screening should include EUS and/or MRI/MRCP, there was no consensus about when to start or end screening.

Risk stratification

Based on personal and family history and genetic testing, patients can be stratified into risk categories. Verna *et al*[181] defined average risk patients as having one family member with PDAC, diagnosed at age 55 or older; these patients do not receive screening with EUS or MRI. Moderate risk patients were defined as those with two or more first, second, or third-degree relatives with PDAC, or an FDR with PDAC diagnosed earlier than age 55; these patients are screened with EUS or MRI. Finally, high risk patients had three or more first, second, or third-degree relatives with PDAC, two or more FDRs with PDAC, one FDR and one SDR with PDAC one of whom was diagnosed before age 55, or a genetic syndrome with PDAC associated with it; these patients receive both EUS and MRI. For all of the risk groups, any abnormal testing is followed by EUS if not already done. Following this screening, if no malignant or premalignant

disease is found, the patient is surveilled based on their risk factors. If malignant or premalignant disease is suspected or diagnosed, surgery must be considered.

Past PDAC screening efforts

A number of PDAC screening programs directed at various high-risk groups have been published, largely focusing on EUS as a screening modality. While each group screened individuals only at elevated risk of PDAC, inclusion criteria, screening modalities, and definition of diagnostic yield varied across groups, resulting in a wide range of reported yields. Their results, with diagnostic yields ranging from 1.1% to 50%, can be found in Table 3[3,9].

CONCLUSION

PDAC is the fourth most common cause of cancer-related deaths in the United States and a major health issue[1]. With dismal five-year survival rates, significant advances in the understanding of the etiology and tumor biology, as well as early detection, screening and treatment of PDAC are needed (Table 4). Given that only those diagnosed at an early or precancerous stage have a reasonable expectation of low morbidity and mortality, increased efforts are needed to improve risk stratification and identify early stage disease or premalignant conditions while they are still resectable. PDAC screening efforts in these enriched cohorts may also allow us to identify more effective modalities for early detection and screening, which could be then modified and instituted in the general population.

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MicroRNAs as emerging biomarkers and therapeutic targets for pancreatic cancer

Marion Gayral, Sébastien Jo, Naima Hanoun, Alix Vignolle-Vidoni, Hubert Lulka, Yannick Delpu, Aline Meulle, Marlène Dufresne, Marine Humeau, Maël Chalret du Rieu, Barbara Bournet, Janick Sèlves, Rosine Guimbaud, Nicolas Carrère, Louis Buscail, Jérôme Torrisani, Pierre Cordelier

Marion Gayral, Sébastien Jo, Naima Hanoun, Alix Vignolle-Vidoni, Hubert Lulka, Yannick Delpu, Aline Meulle, Marlène Dufresne, Marine Humeau, Maël Chalret du Rieu, Barbara Bournet, Janick Sèlves, Rosine Guimbaud, Nicolas Carrère, Louis Buscail, Jérôme Torrisani, Pierre Cordelier, Cancer Research Center of Toulouse Team 10, UMR INSERM U1037, Université Paul Sabatier, 31100 Toulouse, France

Alix Vignolle-Vidoni, Marine Humeau, Maël Chalret du Rieu, Barbara Bournet, Janick Sèlves, Rosine Guimbaud, Nicolas Carrère, Louis Buscail, Pôle Digestif, Centre Hospitalier Universitaire Toulouse, 31100 Toulouse, France

Author contributions: Gayral M, Jo S, Hanoun N, Vignolle-Vidoni A, Lulka H, Delpu Y, Meulle A, Dufresne M, Humeau M, Chalret du Rieu M, Bournet B, Sèlves J, Guimbaud R, Carrère N, Buscail L, Torrisani J and Cordelier P contributed to the bibliographical review; Gayral M, Torrisani J and Cordelier P wrote the paper.

Correspondence to: Pierre Cordelier, PhD, Cancer Research Center of Toulouse Team 10, UMR INSERM U1037, Université Paul Sabatier, Toulouse Oncopole 1, Avenue Joliot Curie, 31100 Toulouse, France. pierre.cordelier@inserm.fr

Telephone: +33-5-61322404 Fax: +33-5-61322403

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Abstract

Despite tremendous efforts from scientists and clinicians worldwide, pancreatic adenocarcinoma (PDAC) remains a deadly disease due to the lack of early diagnostic tools and reliable therapeutic approaches. Consequently, a majority of patients (80%) display an advanced disease that results in a low resection rate leading to an overall median survival of less than 6 months. Accordingly, robust markers for the early diagnosis and prognosis of pancreatic cancer, or markers indicative of survival and/or metastatic disease are desperately needed to help alleviate the dismal prognosis of this cancer. In addition, the discovery of new therapeutic targets is mandatory to design effective treatments. In this review, we will highlight the translational studies demonstrating that microRNAs may soon translate into clinical applications as long-awaited screening tools and therapeutic targets for PDAC.

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Key words: MicroRNAs; Biomarkers; Pancreatic cancer; Therapeutic targets; Precancerous lesions

Core tip: Robust biomarkers and reliable treatments are needed to help alleviate the dismal prognosis of pancreatic cancer. In this review, we will highlight the translational studies demonstrating that microRNAs may soon translate into clinical applications as long-awaited screening tools and therapeutic targets for this cancer.

PANCREATIC CANCER

There are currently no means for the reliable diagnosis of early stages of pancreatic cancer (PDAC) and the curative treatment of late stages. Consequently, the vast majority

of patients (80%) display an advanced disease that results in a low resection rate leading to a dismal overall median survival of less than 6 mo[1]. The estimated 5-year survival rate is lower than 2%. While PDAC is not among the most common tumors, it is one of the most frequent causes of cancer-related death with approximately 40000 death/year in the United States and in Europe. Thus, there is an urgent need to discover diagnostic as well as prognostic molecular markers together with reliable therapeutics to improve pancreatic cancer management.

PDAC is a highly heterogeneous disease[2] defined by numerous alterations in multiple signaling pathways[3]. Additionally, specific cellular clones for primary tumors and metastasis have been identified[4]. Interestingly, the type and number of genomic rearrangements in DNA vary considerably between patients, and occur early during tumor development[5]. On the other end, pioneering studies using genome-wide profiling showed that microRNAs (miRNA) expression can discriminate cancers with high efficacy[6]. In this review, we will focus on the use of miRNAs as promising biomarkers and therapeutic targets for pancreatic cancer (Tables 1-3).

GENERAL CONCEPT OF MIRNAS AND CANCER

miRNAs are small RNA molecules that functions as translation inhibitors of messenger RNA by their binding to 3'-untranslated region[7-9]. These molecules are tightly involved in the regulation of many physiological processes such as development, proliferation, invasion, and apoptosis among others. Interestingly, their expression is profoundly altered in cancer and/or is strongly modulated during carcinogenesis. Thus, the activation of tumor-suppressive miRNAs and the inhibition of oncogenic miRNAs by small molecules or gene transfer may have the potential to provide a fundamentally new approach for the development of cancer therapeutics. Probably the most important advantage in comparison with current approaches targeting single genes is the ability to modulate many different pathways "at once" taking into account that one miRNA can regulate hundreds of genes, frequently in the context of a cell-specific network.

MIRNAS AS DIAGNOSTIC MARKERS FOR PANCREATIC CANCER

To date, many strategies based on high-throughput screening are used to discover relevant clinical biomarkers. For PDAC in particular and pancreatic tissue in general, these protocols are often hindered by the intrinsic high levels of many nucleases. Consequently, the high stability of miRNAs in tissues and fluids is a key advantage over protein and mRNA. In addition, miRNAs can be quantified in very low amounts of material and in highly degraded samples, such as small biopsies and fine needle aspirates. This is mandatory to support the use of miRNAs as biomarkers for PDAC at the clinical level. In the

next sections, we will update the excellent reviews[2,10-15] and meta-analysis[16] from other groups, and reviews and book chapters we recently published[17-19] on the use of miRNAs as biomarkers in PDAC (Tables 1 and 2).

Historically, Pr Schmittgen's group was the first to report the expression profiles of miRNAs in PDAC. They identified miRNAs specifically over expressed in PDAC (miR-376a, miR-301) or in other tumors (miR-155, miR-21, miR-221 and miR-222)[20]. Two additional miRNas (miR-132 and miR-212) were recently reported to be over expressed in PDAC as compared to normal or benign adjacent pancreas to the tumor[21]. Another study by Pr Shao's group yielded conflicting results as they demonstrated that miR-132 was down regulated in cancer *vs* normal benign normal tissues[22]. Other miRNAs, such as miR-96[23], miR-34a[24] and miR-21[25], have been reported to be altered in PDAC as compared to normal adjacent tissue. miRNAs expression may also help to discriminate PDAC from chronic pancreatitis. This is of particular importance to prevent from unnecessary and possibly debilitating surgery, or to delay tumor treatment, respectively. Historically, Pr Bloomston's group reported that 21 miRNAs with increased expression and 4 underexpressed miRNAs differentiated PDAC from normal tissue in 90% of samples and from pancreatitis with 93% accuracy[26]. Twenty additional miRNAs were discovered by Szafranska *et al*[27] to discriminate between PDAC, chronic pancreatic and normal pancreas. Later, expression of miR-203[28], miR-148a[29], miR-196b[29], miR-196a[29] and miR-205[29] were demonstrated to be altered in PDAC *vs* chronic pancreatitis. Alternatively, miRNA expression profiles have been recently used to distinguish PDAC from cholangiocarcinoma, two virtually indistinguishable cancers using conventional histopathological and clinical characteristics[30].

Endoscopic ultrasound-guided fine needle aspirations (FNA) material allows for the screening of the vast majority (> 85%) of PDAC patients that are not eligible for surgery, and, as consequence, may provide new insights for the diagnosis and prognosis of PDAC. Pr Szafranska's group was the first to demonstrate that the expression of miR-196a and miR-217 in FNA material can classify PDAC from benign lesion[31]. This pioneering study led to the development of the first molecular test for the identification of PDAC[32]. Hence, we demonstrated that *let-7* miRNA expression is repressed in PDAC FNAs[33], and that the measurement of hypermethylation of miR-148a encoding DNA region is potentially useful to differentiate PDAC and pseudo-tumor forms of chronic pancreatitis[34].

MIRNAS AS PROGNOSTIC AND PREDICTIVE MARKERS FOR PANCREATIC CANCER

MiRNAs are also scrutinized for their ability to predict cancer prognosis and/or response to treatment. Bloomston *et al*[26] were the first to report that miR-452,

Table 1	MicroRNAs as diagnostic markers for pancreatic cancer			
miRNA	Biopsies	FNA	Circulating	Ref.
Let-7a	X (↓)	X (↓)		[33]
miR-34a	X (↓)			[24]
miR-96	X (↓)			[23]
miR-99a			X (↑)	[55]
miR-101	X (↑)			[67]
miR-132	X (↑/↓)			[21,22]
miR-141	X (↓)			[27]
miR-143	X (↑)			[27]
miR-145	X (↑)			[27]
miR-146a	X (↑)			[27]
miR-148a	X (↓)			[27,29]
miR-148b	X (↓)	X (↓)		[27,34]
miR-150	X (↑)			[27]
miR155	X (↑)		X (↑)	[26,27,53,62,67,68]
miR-16			X (↑)	[57]
miR-181a	X (↑)			[26]
miR-181b	X (↑)			[26]
miR-181d	X (↑)			[26]
miR-185			X (↑)	[59]
miR-191			X (↑)	[55,59]
miR-196a	X (↑)	X (↑)	X (↑)	[27,29,32,53,57]
miR-196b	X (↑)			[27,29]
miR-20a			X (↑)	[55,59]
miR-200a			X (↑)	[56]
miR-200b			X (↑)	[56]
miR-203	X (↑)			[28]
miR-210	X (↑)		X (↑)	[29,54]
miR-212	X (↑)			[20]
miR-216	X (↓)			[27]
miR-217	X (↓)	X (↓)		[27,31]
miR-21	X (↑)	X (↑)	X (↑)	[20,26,25,51,53,55,60,64, 67-69]
miR-210	X (↑)		X (↑)	[27,53]
miR-221	X (↑)		X (↑)	[20,26,58,69]
miR-222	X (↑)			[20,26,27]
miR-223	X (↑)			[27]
miR-24			X (↑)	[55]
miR-27a-3p			X (↑)	[59]
miR-29c	X (↓)			[27]
miR-30a-3p	X (↓)			[27]
miR-301	X (↑)			[20]
miR-31	X (↑)			[27]
miR-375	X (↓)			[27]
miR-376a	X (↑)			[20]
miR-494	X (↓)			[27]
miR-1290	X (↑)			[61]

miRNAs: MicroRNAs; Biopsies: Resected tumors; FNA: Fine needle aspiration; ↑: Upregulated; ↓: Downregulated.

miR-105, miR-127, miR-518a-2, miR-187, and miR-30a-3p are over-expressed in the tumors of patients with survival greater than 2 years. Moreover, tumors with high expression of miR-196a-2 or miR-219 have a lower median survival compared with those with low expression. In addition, over expression of miR-155[35], miR-200[35], miR-203[35], miR-205[35], miR-200c[36], miR-21[37], miR-212[38] and miR-675[38] and reduced expression of miR-34a[37], miR-30d[37], miR-148a[38], miR-187[38], miR-130b[39] and let-7g[38] in PDAC are associated with poorer survival rate. Last, low miR-211 expression was demonstrated as an independent factor of poor prognosis in resected PDAC[40].

Gemcitabine is broadly used as a first-line chemotherapeutic treatment for patients with unresectable locally advanced or metastatic pancreatic cancer[41]. However, the 5-year survival rate is only 2%[42], with 1-year survival rates ranging from 17% to 23%[41]. Recently, phase Ⅱ and Ⅲ trials exploring gemcitabine-based combinations with erlotinib[43], FOLFIRINOX[44] or nab-Paclitaxel[45] were found to improve overall survival of patients. However, the moderate activity of standard gemcitabine and gemcitabine-based regimens still encourages the discovery of robust biomarkers that may help to stratify PDAC patients for tailored therapy. Gemcitabine requires transporter proteins to cross cell membranes. Low expression of human equilibrative nucleoside transporter-1 (hENT1) may result in gemcitabine resistance in PDAC. Recent studies have revealed that high levels of hENT1 in PDAC predict longer survival times in patients treated with adjuvant gemcitabine[46]. In another study, CO-101, a lipid-drug conjugate of gemcitabine, was designed to enter cells independently of hENT1[47]. However, CO-101 was found not superior to gemcitabine in patients with metastatic PDAC and low tumor hENT1. In addition, metastasis hENT1 expression doesn't predict gemcitabine outcome. Interestingly, Giovannetti *et al*[48] found that high miR-21 expression in tumors is associated with shorter overall survival both in the metastatic and in the adjuvant setting, while patients with low miR-21 expression may benefit from gemcitabine treatment[49]. Gemcitabine resistance is also associated with the cellular over expression of miR-146 and the reduced expression of miR-205 and miR-7[50]. Last but not least, Pr Korc's group recently demonstrated that miR-10b is a novel and powerful diagnostic biomarker for PDAC[51]. Like miR-21, miR-10b is over expressed in the FNA material from PDAC patients. Additionally, reduced expression of miR-10b is associated with improved response to multimodality neoadjuvant therapy, likelihood of surgical resection, delayed time to metastasis, and increased survival. Thus, miR-10b is likely to be a novel marker to diagnose PDAC, but may also serve as a biomarker for response to gemcitabine-based neoadjuvant therapy, and be predictive of early metastasis formation. In experimental models, miR-10b was demonstrated to promote PDAC-derived cells proliferation and invasion by suppressing TIP30, which enhances EGFR signaling, facilitates EGF-TGF-β cross-talk together with the expression of epithelial-mesenchymal transition-promoting genes[52].

CIRCULATING MIRNAS AS BIOMARKERS FOR PANCREATIC CANCER

The recent discovery of miRNAs in serum or plasma opens up the possibility of using non coding RNAs as circulating biomarkers of disease. Wang *et al*[53] were the first to report the detection of miRNA in the blood of PDAC patients. They demonstrated that plasmatic miR-21, miR-210, miR-196a and miR-155 reveal a sensi-

Table 2 MicroRNAs as prognostic and predictive markers for pancreatic cancer

miRNA	Biopsies	FNA	Prognostic	Predictive of treatment efficacy	Ref.
miR-105, miR-127, miR-187, miR-30a-3p, miR-452, miR-518a-2	X (↑)		+		[26]
miR-155, miR-200, miR-203, miR-205	X (↑)		+		[35]
miR-21 (↑), miR-34a (↓), miR-30d (↓)	X		-		[37]
miR-212 (↑), miR-675 (↑), miR-148a (↓), miR-187 (↓), let-7g (↓)	X		-		[38]
miR-146 (↑), miR-205 (↓), miR-7 (↓)	X		-	-	[50]
miR-10b		X (↑)		-	[51,52]
miR-196a	X (↑)		-		[26]
miR-219	X (↑)		-		[36]
miR-200c	X (↑)		+		[36]
miR-21	X (↑)	X (↑)	-	-	[40,41,51]

miRNAs: MicroRNAs; Biopsies: Resected tumors; FNA: Fine needle aspiration; ↑: Upregulated; ↓: Downregulated; +: Good prognosis/response to treatment; -: Bad prognosis/response to treatment.

Table 3 MicroRNAs as therapeutic targets in pancreatic cancer

miRNA	Expression	Known target(s)	Function	Ref.
Let-7	↓	KRAS	Inhibition of cell proliferation, chemosensitization	[33,85,86]
miR-10b	↑	TIP30	Increased cell proliferation and invasion	[52]
miR-21	↑		Inhibition of cell proliferation, invasion, tumor growth, chemoresistance and inhibition of apoptosis	[95]
miR-23b			Radioresistance	[91]
miR-29a		Wnt/β-catenin	Chemosensitization to gemcitabine	[88]
miR-34a	↓	Smad3	Inhibition of cell proliferation and invasion, induction of apoptosis	[82,83,94]
miR-96	↓	KRAS	Inhibition of cell proliferation, invasion, tumor growth and induction of apoptosis	[23,70]
miR-99b		mTOR	Radioresistance	[90]
miR-132	↑↓	Rb1	Alteration of cell proliferation	[31,32]
miR-138		lipocalin	Inhibition of tumorigenicity	[93]
miR-141	↓	MAP4K4	Inhibition of cell proliferation and invasion, chemosensitization	[92]
miR-142-3p			Inhibition of cell proliferation	[72]
miR-148a	↓		None	[78]
miR-148b	↓	AMPKα1	Inhibition of cell proliferation, invasion and chemosensitization	[79]
miR-150*		IGF-1R	Induction of apoptosis	[73]
miR-181b	↑	NFkB	Chemosensitization to gemcitabine	[89]
miR-197			Induction of EMT	[75]
miR-198	↓	MSLN, OCT-2, PBX-1, VCP	Inhibition of cell proliferation, invasion, tumor growth and induction of apoptosis	[71]
miR-212	↑	Rb1	Increased cell proliferation	[21]
miR-218	↓		Inhibition of cell proliferation and tumor growth and metastasis	[84]
miR-221	↑		Increased migration, proliferation and EMT	[77]
miR-320c		SMARCC1	Chemosensitization to gemcitabine	[77]
miR-373			Increased tumor growth	[74]
miR-630		IGF-1R	Induction of apoptosis	[73]
miR-655			Inhibition of EMT	[76]

↑: Upregulated; ↓: Downregulated; EMT: Epithelial-mesenchymal transition.

tivity of 64% and a specificity of 89% for PDAC. A recent study further confirmed that circulating miR-210[54] and miR-21[55] are elevated in PDAC patients and may potentially serve as a useful biomarker for PDAC diagnosis. In addition, miR-200a[56], miR-200b[56], miR-16[57], miR-196a[57], miR-20a[55], miR-24[55], miR-25[55], miR-99a[55], miR-185[55], miR-221[58] and miR-191[55] were described as significantly elevated in the sera of PDAC as compared with controls. Combining miR-16[57] and miR-196a[57], or miR-27a-3p[59] detection with CA 19-9 quantification is even more effective to discriminate PDAC from controls. However, Pr Hoheisel's group recently reported that blood miRNAs profile could not discriminate pancreatitis from PDAC efficiently[60]. Last, but not least, Pr Goggin's group recently demonstrated that miR-1290 accurately distinguishes patients with low-stage pancreatic cancer from healthy and disease controls[61]. Such study paves the way for the non-invasive detection of early PDAC lesions.

OPEN QUESTION: WHAT IS THE SIGNIFICIANCE OF MIRNAS IN HIGH RISK PATIENTS FOR DEVELOPING PANCREATIC CANCER?

One of the current avenues of research to improve the management of pancreatic cancer is to better understand the early stages of the disease in order to allow for curative surgery and to prevent the risk of cancer in popula-

tions at risk. Advances in biomedical research have led to recent evidence that pancreatic cancer develops from preneoplastic lesions which can be considered as very effective risk factors. Three types of lesions have been identified so far: pancreatic intraepithelial neoplasia, mucinous cystadenomas, and Intraductal Papillary Mucinous Neoplasia of the pancreas (IPMN). Interestingly, the latter lesions can be readily detected due to the progress and the multiplicity of the imaging devices in the clinical departments. The risk of degeneration of IPMNs varies according to the type of injured duct: it is of the order of 60% for IPMN located in the main duct (or mixed) while this risk is estimated at 15% for branch ducts. IPMN now represent 25% of the diagnosed pancreatic cystic tumors and 20% of resected pancreatic tumors, respectively. Therefore, one of the most promising strategies to improve the dismal prognosis of pancreatic cancer is to identify early indicators of degeneration of IPMNs in populations at high risk of developing this cancer. Interestingly, miRNAs have recently revealed a great potential as reliable early diagnosis biomarkers in IPMNs. Again, miR-21 and miR-155 are highly expressed in IPMN, while miR-155 is elevated in IPMN-associated pancreatic juice as compared to controls[62]. We demonstrated that miR-205 and miR-21 overexpression precede phenotypic changes in the pancreatic ducts, both in human samples and in transgenic mice developing cancer[63]. Interestingly, such over expression may occur early in the transformation from normal pancreatic tissue, as benign cystic tumors of low and high malignant potential express high levels of this miRNA[64]. This strongly suggests that miRNAs such as miR-21 can possibly be used for an early diagnosis of this neoplasm. In a similar experimental model, Yabushita *et al*[65] recently reported the over expression of miR-155, miR-21, miR-210, miR-18a, miR-203, miR-30b-5p, miR-31, miR369-5p, miR3-376a and miR-541 in the serum of a human KRAS oncogenic transgenic rat model. More importantly, Matthaei *et al*[66] assessed the diagnostic benefit of using miRNAs as biomarkers in pancreatic cyst fluid in patients, to identify IPMN that require resection and exclude non-mucinous cysts with a sensitivity of 89%, a specificity of 100%, and AUC of 1. This work was further completed by Pr Giovannetti's group who demonstrated that miR-21, miR-155 and miR-101 showed significant differences in invasive *vs* non-invasive IPMNs, with miR-21 described as an independent prognostic biomarker in invasive IPMNs[67]. Again, miR-21 and miR-155 were recently described as upregulated during the development and progression of IPMN[68]. MiR-21 in cystic fluid was identified as a candidate biomarker to distinguish between benign, premalignant, and malignant cysts[69], while miR-221 could be used for the identification of more advanced malignant disease[69]. Last, a work from Pr Maitra's group recently revealed that a 9-miRNA panel quantified in cystic fluid may aid in diagnosis and surgical treatment decisions for patients with pancreatic cystic lesions, such as high-grade IPMNs[66]. Thus, miRNAs may reveal as non-invasive indicators of degeneration in a population at high risk of

developing incurable cancer. Once identified, patients will be stratified and will benefit from early surgical management that will greatly improve their survival and prognosis. Finally, this approach is likely to strengthen the surveillance protocol and to reduce the costs associated with patients care.

ROLE OF MIRNAS IN PANCREATIC CANCER

miRNAs are broadly involved in pancreatic carcinogenesis

Many miRNAs have been reported to alter cancer proliferation and/or migration, both *in vitro* and *in vivo*. miR-132 and miR-212 were recently reported to be over expressed in pancreatic cancer as compared to normal or benign adjacent pancreas to the tumor[21]. Interestingly, these miRNAs target the retinoblastoma tumor suppressor 1 (Rb1) to favor cancer cell proliferation[21]. Another study by Pr Shao's group yielded conflicting results as they demonstrated that miR-132 was down regulated in cancer *vs* normal benign normal tissues[22]. In the later study, enforced expression of miR-132 in cell lines derived from PDAC led to proliferation and colony formation inhibition[22]. Yu *et al*[23] reported that miR-96 is downregulated in PDAC as compared to normal tissues and targets KRAS. Consequently, restoring miR-96 expression strongly inhibited *in vitro* cell proliferation, invasion, induced apoptosis and reduced tumor growth. This was further confirmed in a recent study linking ecotropic viral integration site 1 oncoprotein-mediated inhibition of miR-96 to promote KRAS expression during early pancreatic carcinogenesis[70]. MiR-198 acts as a central tumor suppressor in PDAC and modulates the expression of many oncogenic factors such as MSLN, OCT-2, PBX-1, and VCP[71]. Very interestingly, low miR-198 expression prognosticates poor patient outcome, while high miR-198 may disrupt this oncogenic network and predict better prognosis and increased survival.

Epigenetic regulation of miRNAs involved in pancreatic cancer progression

MiR-148 family members may have distinct effects on PDAC-derived cells proliferation. While miR-148a expression is lost during PDAC carcinogenesis following methylation of its DNA sequence[34], we recently demonstrated that enforced expression of this miRNA didn't impaired PDAC-derived cells cell proliferation nor tumor growth in experimental models[72]. On the other hand, recent results described that miR-148b can inhibit cell proliferation, invasion, and enhance chemosensitivity of PDAC by targeting AMPKα1[73]. MiR-124 is also silenced by aberrant methylation in PDAC; consequently, tumor progression and metastasis are enhanced due to the lack of Rac1 targeting[74]. MiR-34a miRNA, which is directly regulated by p53, is also subjected to epigenetic silencing in numerous neoplasms, including PDAC[75]. Strikingly, this miRNA plays a pivotal role in PDAC stem cell self-

renewal and may hold significant promise as novel target for PDAC[24]. In addition, the natural compound genistein up-regulates this miRNA to suppress cell proliferation and induce cell death by apoptosis of PDAC-derived cell lines[76]. MiR-34a was also recently reported as a tumor metastasis suppressor by negatively modulating Smad3[77]. Last, Li *et al*[78] recently demonstrated that the histone methyltransferase enhancer of zeste homolog 2 inhibits miR-218 expression, that prevents proliferation of PDAC cells in culture, and tumor growth and metastasis in nude mice.

miRNAs regulates the epithelial-mesenchymal transition in pancreatic cancer

Besides the miR-200 family members (reviewed elsewhere), miRNAs such as miR-197 and miR-655 have been recently involved in the epithelial-mesenchymal transition in PDAC cells, by targeting p120 catenin[79] and ZEB1 and TGFBR2, respectively[80]. In addition, microRNA-221 participates in the effects of PDGF-BB on migration, proliferation, and to the epithelial-mesenchymal transition in these cells[81].

miRNAs are key players in drug-mediated inhibition of pancreatic cancer growth

Recently, different molecules were found to alter miRNA expression in PDAC to inhibit cell proliferation and/or tumor growth. Triptolide that downregulates HSP70, a molecular chaperone upregulated in several tumor types, was recently shown to upregulate miR-142-3p in PDAC cells, to inhibit cell proliferation[82]. More importantly, Minnelide, a water-soluble prodrug of triptolide, induces the expression of miR-142-3p *in vivo*. In addition, the adamantyl retinoid-related molecule 3-Cl-AHPC was recently demonstrated to induce miR-150* and miR-630 miRNAs expression to target IGF-1R and promote apoptosis in PDAC cells[83]. Inappropriate regulation of intracellular zinc levels may also plays an important role in PDAC. Recently, increased zinc influx mediated by the zinc importer ZIP4 was demonstrated to induce miR-373 expression in pancreatic cancer to promote tumor growth[84].

Besides miR-148b, *Let-7* is also involved in the chemosensitization of PDAC-derived cell lines. Indeed, reduced expression of the *let-7* miRNAs family members was identified in gemcitabine-resistant PDAC cell lines[85]. This was correlated with a higher expression of ribonucleotide reductase subunit M2 (RRM2), a key protein involved in gemcitabine resistance. In this work, the authors nicely demonstrated that *Let-7* can regulate RRM2 expression, but also that *Let-7* biogenesis was severely impaired in PDAC cells[85]. The latter effect seems to be recurrent in PDAC as nuclear TRAILR2 was recently demonstrated to inhibit maturation of *Let-7* in PDAC cell lines to increase their proliferation[86]. Additionally, miR-320c, miR-29a and miR-181b were found to regulate the resistance of PDAC cells to gemcitabine through SMARCC1[87], the Wnt/β-catenin[88] and the

NF-κB[89] signaling pathways, respectively. In a recent report, miR-141 was found to target MAP4K4 to inhibit cell proliferation, clonogenicity and invasion, induce G1 arrest and apoptosis, and enhance chemosensitivity[90]. Alternatively, radiation resistance of PDAC-derived cell lines has also been linked to miRNAs, such as miR-99b[91]. In a very interesting study by Wang *et al*[92], miR-23b was found to regulate autophagy associated with radioresistance of PDAC cells.

MIRNAS AS NEW THERAPEUTIC TARGETS FOR PANCREATIC CANCER MANAGEMENT

As stated in the previous sections, miRNA expression is profoundly altered in pancreatic cancer and/or is strongly modulated during carcinogenesis. Thus, the activation of tumor-suppressive miRNAs and the inhibition of oncogenic miRNAs may have the potential to provide a fundamentally new approach for the development of therapeutics for many cancers including PDAC. Probably the most important advantage in comparison with current approaches targeting single genes is the ability to modulate many different pathways "at once" taking into account that one miRNA can regulate hundreds of genes, frequently in the context of a cell-specific network. In this section, we will update our recent book chapters on the use of miRNAs as therapeutic tools to control PDAC progression[17,18] (Table 3).

Few reports described the use of miRNAs as therapeutic targets to control PDAC tumor progression, *in vivo*. We demonstrated that *let-7* enforced expression strongly inhibits PDAC cell proliferation[33]. This was achieved either using plasmid-encoding miRNA or lentiviral vectors. However, restoring *let-7* levels in cancer-derived cell lines failed to impede tumor growth progression after intratumoral gene transfer. Using a similar strategy, Lee *et al*[93] recently demonstrated that miR-138 transfection of cancer cells *in vivo* reduces tumor formation by targeting neutrophil gelatinase-associated lipocalin. Interestingly, nanoparticles targeted to PDAC-derived cells using bifunctional CC9 peptide successfully delivered miR-34a to inhibit the growth of subcutaneous PANC-1 tumors[94]. We recently devised a lentiviral vector to target miR-21, one of the most described miRNA in oncology[95]. Following transduction with this vector, PDAC-derived cells cell proliferation is strongly inhibited, and cancer cells die by apoptosis through the mitochondrial pathway. *In vivo*, a single inoculation of the therapeutic vectors in exponentially growing PDAC tumors stops cancer progression, inhibits cell proliferation and provokes cancer cell death by apoptosis. We found that our approach surpasses the therapeutic efficacy of standard treatments for this disease. Interestingly, miR-21 depletion enhances tumor angiogenesis; consequently, combining miR-21 targeting with gemcitabine eradicate experimental PDAC tumors. During this study, we treated existing tumors with miR-21

antagonists, a paradigm closely related to the clinical scenarios in which such therapies will be employed. While there clearly remains significant work to be done, this work is the first to demonstrate that targeting oncogenic miRNA is very effective to stop the tumor growth of a very aggressive PDAC model. It also emphasizes the central role of miR-21 in this cancer, and paves the way to forthcoming studies to discover the many pathways controlled by this miRNA in PDAC. Because miR-21 is over expressed in most human tumors; therapeutic delivery of miR-21 antagonists may still be beneficial for a large number of cancers for which no cure is available.

CONCLUSION

miRNAs can be detected and quantified not only in frozen tissues, but also in formalin-fixed paraffin-embedded tissues, as well as serum and plasma samples. These tiny but potent molecular markers have proven effective for PDAC classification, prognostic stratification and drug-response prediction. Strikingly, miR-21, and to a lower extent miR-196, miR-217, miR-10b and miR-155, appears to be constantly up regulated in PDAC, and to be indicative of poor survival, response to treatment and/or metastatic disease. PDAC is also frequently associated with a dense stromal reaction that may favor tumor progression and resistance to treatment. Recently, Pr Donahue's group has pointed out that miR-21 expression in PDAC tumor-associated fibroblasts is associated with decreased overall survival and promotes tumor cells invasion[96]. This work may stem for novel diagnostic and therapeutic strategies for dual targeting of both tumor and stroma in PDAC. Whether this will translate into clinical applications is still highly debated. Above all, circulating miRNAs, in combination with other "omics" approaches such as proteomics, are expected in the future to prove specific and/or sensitive as a long-awaited screening tool for PDAC.

On the other hand, miRNAs are key players in PDAC carcinogenesis, and can be organized in oncogenic networks aimed at inhibiting multiple tumor suppressor genes. They are involved in the regulation in many if not all cancerous pathways such as cell proliferation, dissemination, resistance to apoptosis or chemotherapy. Consequently, the development of miRNA-based therapies have the potential to overcome the limitations of present cancer therapies that often lead to relapse because of the complexity and the redundancy of the targeted signaling pathways. The path from drug discovery to clinical trials is long and still hampered by many challenges. Despite the fact that hundreds of ongoing clinical trials include miRNA as biomarkers, miR-122 is the unique miRNA that as successfully reached clinical trial as targeted therapy to treat HCV infection[97,98]. Nevertheless, it is our belief that miRNA-based therapeutics (especially to target miR-21) for cancer are not far behind, and that combination of miRNA therapy with targeted or traditional therapies may provoke a synergistic effect for treatment

of cancer in clinical trials in the next few years.

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2014 ADVANCES IN PANCREATIC CANCER

Selection criteria in resectable pancreatic cancer: A biological and morphological approach

Domenico Tamburrino, Stefano Partelli, Stefano Crippa, Alberto Manzoni, Angela Maurizi, Massimo Falconi

Domenico Tamburrino, Stefano Partelli, Stefano Crippa, Alberto Manzoni, Angela Maurizi, Massimo Falconi, Pancreatic Surgery Unit, Department of Surgery, Polytechnic University of Marche Region, 60126 Ancona-Torrette, Italy
Author contributions: Crippa S, Manzoni A and Maurizi A performed the review of the literature; Tamburrino D and Partelli S wrote the paper under the supervision of Falconi M.
Correspondence to: Massimo Falconi, MD, Pancreatic Surgery Unit, Department of Surgery, Polytechnic University of Marche Region, Via Conca 71, 60126 Ancona-Torrette, Italy. m.falconi@univpm.it
Telephone: +39-71-5965781   Fax: +39-71-5964429
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Core tip: The aim of this work was to improve identification of patients with pancreatic ductal adenocarcinoma, who will benefit from pancreatic resection. Duration of symptoms and level of carbohydrate antigen 19.9 in patients with resectable disease should be considered to avoid R1 resection and early relapse. Radiological assessment can help surgeons to distinguish resectable disease from borderline resectable disease and locally advanced pancreatic cancer.

Abstract

Pancreatic ductal adenocarcinoma (PDA) remains one of the most aggressive tumors with a low rate of survival. Surgery is the only curative treatment for PDA, although only 20% of patients are resectable at diagnosis. During the last decade there was an improvement in survival in patients affected by PDA, possibly explained by the advances in cancer therapy and by improve patient selection by pancreatic surgeons. It is necessary to select patients not only on the basis of surgical resectability, but also on the basis of the biological nature of the tumor. Specific preoperative criteria can be identified in order to select patients who will benefit from surgical resection. Duration of symptoms and level of carbohydrate antigen 19.9 in resectable disease should be considered to avoid R1 resection and early relapse. Radiological assessment can help surgeons to distinguish resectable disease from borderline resectable disease and locally advanced pancreatic cancer. Better patient selection can increase survival rate and neoadjuvant treatment can help surgeons select patients who will benefit from surgery.

INTRODUCTION

Despite recent advances in cancer therapy, pancreatic ductal adenocarcinoma (PDA) remains one of the most aggressive tumors and is among the four most frequent causes of tumor-associated deaths in both men and women in the European Union and the United States[1,2]. Surgical resection still represents the only curative treatment for PDA, although only a small fraction of tumors is amenable to surgical resection at diagnosis[3-6]. Moreover, among patients who undergo surgery, 30% develop early recurrence as a result of misdiagnosed aggressive disease[6]. The aim of this paper is to review the current available data on factors related to adverse prognosis in

patients with resectable PDA.

EPIDEMIOLOGY

Only 20% of patients with PDA are resectable at diagnosis and 5-year overall survival (OS) after curative resection is only 20%[4-8]. During the last decade survival rates of PDA have remained dismal with a 5-year OS of 15%-20% after pancreaticoduodenectomy and 8%-15% after distal pancreatectomy[9,10]. In the 1990s there was no improvement in 5-year OS, which was even lower (2.3%-2.7%) compared with the 5-year OS rate observed in the late 1980s (2.5%-3.1%)[11]. Despite progress in diagnostic procedures, most cases are still metastatic at diagnosis, and are not amenable to radical surgery and even when curative surgery is performed, most patients will eventually relapse[11]. In a large, retrospective, study of a high-volume centre in Italy, Barugola *et al*[12] compared the survival time-trends in a selected population of patients affected by resectable PDA. There were 114 (21%) resections in 1990-1999 and 430 (79%) in 2000-2008. The length of hospital of stay (16 d *vs* 10 d) and postoperative mortality (2.6% *vs* 1.1%) significantly decreased over time. The median disease-specific survival significantly increased from 16 mo in the first period to 29 mo in the second period. Resection performed in 1990-2000 was an independent predictor of poor outcome, indicating that long-term survival after surgery for resectable PDA significantly improved in the last decade. This improvement is possibly explained by the advances in cancer therapy but also by better patient selection by pancreatic surgeons. As regards oncological progress, in recent years several efforts have been made to develop effective drugs for pancreatic cancer. In particular, two recent randomized clinical trials that included patients with metastatic PDA demonstrated significantly better survival for the treatment groups compared with control groups of patients treated with gemcitabine[13,14]. Conroy *et al*[14] showed that patients treated with FOLFIRINOX (5-fluorouracil, oxaliplatin, and irinotecan) had improved survival compared with a gemcitabine alone group, with a median OS of 11.1 mo *vs* 6.8 mo with an objective response rate of 31.6% *vs* 9.4%. Similarly, Von Hoff *et al*[13] have shown a better survival in patients with PDA treated with gemcitabine plus nab-paclitaxel compared with gemcitabine alone. In this work, OS was 8.5 mo in the treatment group compared with 6.7 mo in the control group. The increase in objective response rate due to improvement in oncological treatments can also have the consequence of increasing the number of resectable patients[15]. Better patient selection has probably modified the survival of patients with PDA because of changing resectability criteria. Among those who undergo surgical resection, up to 30% of patients die of disease within 1 year after surgery[6,16]. In this subgroup, recurrence is early, and survival rates are comparable to those observed in patients with advanced disease undergoing antitumoral therapies alone[17]. The risk of early failure

after surgery could be associated with the following: (1) inadequate preoperative radiological staging; (2) lack of radical surgery; and (3) differences in tumor aggressiveness. Undoubtedly, what is common to patients who will recur early, is disease with more aggressive biological behavior.

All of these patients are resectable at diagnosis, but probably the difference with the others patients is the biological characteristics of the tumor. In addition, there is a relationship between hospital volume with long-term survival in patients with cancer subjected to pancreatectomy, probably due to patient selection and technical expertise at the major centers that are responsible[18]. Therefore, it is necessary to select patients not only on the basis of surgical resectability, but also on the basis of the biological nature of the tumor.

Preoperatively, we can identify specific criteria to be recognized in order to select those patients who will actually benefit from surgical resection. Focusing on these criteria, we suggest a step-by-step approach for patients with pancreatic cancer; the first step is to consider their clinical and laboratory factors and then their radiological features.

CLINICAL AND LABORATORY CRITERIA

In order to select patients who will benefit from a surgical approach, we have to consider not only the imaging but also other parameters such as symptoms, risk of mortality related to the patient's comorbidity, and the level of carbohydrate antigen (CA),19.9. Symptoms of PDA depend on the site of the pancreatic lesion; for pancreatic head tumors, jaundice is the first sign, whereas for pancreatic body/tail tumors, pain is the most frequent symptom. Duration of symptoms > 40 d is an important parameter associated with a higher risk of early recurrence among patients who undergo surgery[6]. Although the reason behind abdominal pain in PDA remains unclear, it is likely that this represents the result of pancreatitis or tumoral invasion of the retroperitoneal nerves[10,19,20]. The presence of invasion of the retroperitoneal nerves, which causes pain, means that the tumor is over the gland, thus, despite radiological resectability, it should be considered as a borderline or locally advance disease. Nevertheless, not all patients with a resectable PDA are also fit for surgery. Before planning pancreatic resection therefore, it is mandatory to assess carefully the surgical risk of each patient. Several studies have demonstrated that elderly patients have an increased risk of morbidity after pancreaticoduodenectomy (PD), in particular related to postoperative pancreatic fistula, although morbidity and mortality rates are acceptable[21]. It could be therefore justified to offer PD to elderly patients who do not have significant comorbidity[21]. Brozzetti *et al*[22] have compared two group of patients (Group A > 70 years and Group B < 70 years). They showed significantly higher operative morbidity and mortality in Group A and they concluded that, although an aggressive surgical approach is justified

in elderly patients with pancreatic adenocarcinoma, surgical complications that lead to reoperation are responsible for high mortality in elderly patients. In addition to general causes, such as concomitant disorders, reduced functional reserve, poor tolerance to stress, and the texture of the pancreatic remnant, there are specific prognostic factors affecting pancreaticojejunostomy leakage and related mortality.

Another important parameter related to the aggressiveness of disease is the level of CA19.9. CA19.9 has been used for the diagnosis, prognosis, and follow-up of pancreatic cancer patients. Preoperative CA19-9 is strongly associated with tumor stage. A decrease in CA19.9 level is the best index of improved prognosis[23,24]. In contrast, patients with increased CA19.9 after resection had a significantly shorter median survival time. In another study published by Montgomery *et al*[25], patients who had CA19.9 < 180 U/mL in the first 3 mo after surgery had improved survival. Lower preoperative CA19.9 values correlated not only with a lower pathological stage, but also with increased post-resection survival. The presence of preoperative CA19.9 < 1000 U/mL was associated with a median survival of 28 mo compared with 12 mo in patients with CA19.9 > 1000 U/mL[23]. CA19.9 > 200 U/mL in patients with resectable PDA is associated with a higher risk of early failure after resection for pancreatic cancer. The importance of CA19.9 levels as a prognostic marker in PDA has been demonstrated in several other studies that have evaluated the decrease in CA 19-9 after anti-tumor therapy. Yang *et al*[26] have shown that patients who had a CA19.9 decrease of > 90% following chemoradiotherapy (CRT), had a significantly improved median survival compared with those who had not (16.2 mo *vs* 7.5 mo). The median survival of patients with a CA19.9 level lower than the median post-CRT value was 10.3 mo, compared with 7.1 mo for those with a CA19.9 level greater than the median. After CRT, CA19.9 < 50 U/mL also had a meaningful prognostic significance. In the neoadjuvant therapy setting, the measurement of CA19.9 is an essential variable in the evaluation of possible surgical resection of tumors that exhibit a response to treatment.

RADIOLOGICAL CRITERIA

The diagnostic phase and the resectability assessment of PDA should always involve a multidisciplinary evaluation. In this setting, it is important to offer patients the expertise of a high-volume center and dedicated multidisciplinary team (MDT). The importance of MDTs has been widely demonstrated for other malignancies[27,28]. Similarly, Pawlik *et al*[29] have analyzed the impact of MDTs in the management of patients with pancreatic cancer. They analyzed 203 patients with computed tomography (CT) that revealed locally advanced/unresectable disease (35%), metastatic disease (18%), and locally advanced disease with metastasis (1%). After an accurate review of the imaging, the clinical stage of the disease was modified in

19% of patients. Overall, 48 out of 203 (24%) patients had a change in their recommended management based on clinical review of their case by the pancreatic MDT. As a consequence, the quality of imaging as well as the expertise of radiologists contributes significantly to better patient selection. Imaging should include at least one high-quality technique such as CT or magnetic resonance imaging. CT should be performed according to a defined pancreas protocol such as triphasic cross-sectional imaging and thin slices. Optimal multiphase imaging techniques include a non-contrast phase, plus arterial, pancreatic parenchymal and portal venous phases of contrast enhancement with thin cuts (3 mm) through the abdomen[30]. The arterial phase shows excellent opacification of the celiac axis and the superior mesenteric artery, whereas the superior mesenteric, portal and splenic veins and the pancreas itself are opacified in the venous phase. Likewise, the detection of liver metastasis is optimal in the latter phase. Weg *et al*[31] and Kopka and Grabbe[32] have noted that a slice thickness of 2-4 mm is superior to 5-10 mm in the detection of small liver metastases. Moreover, the introduction of multidetector CT imaging has allowed the acquisition of these thinner slices in liver imaging, resulting in improved detection rates of liver metastases[33]. Vascular involvement is another important finding that can be assessed preoperatively by CT scan. A classification of vascular involvement in pancreatic cancer has been defined by the MD Anderson Group[34]. This classification includes two separate entities: (1) borderline resectable: PDA that is defined as a tumor with an abutment ≤ 180° (one half or less) of the circumference of the superior mesenteric artery (SMA) and/or with a short-segment encasement/abutment of the common hepatic artery (typically at the gastroduodenal origin) and/or with short-segment occlusion with suitable vessel above and below in superior mesenteric vein (SMV) or portal vein (PV); and (2) locally advanced: PDA that is defined as a tumor with an encasement > 180° of the SMA and/or with an encasement and no technical option for reconstruction usually because of extension to the celiac axis/splenic/left gastric junction or the celiac origin, and/or with occlusion of the SMV/PV without an option for reconstruction. Nonoperative management for locally advanced pancreatic cancer (LAPC) is largely accepted[15,35-37]. Neoadjuvant treatment with combination chemotherapy results in a higher resection rate compared with single agent chemotherapy (33% *vs* 27%) as confirmed by Gillen *et al*[38] in their meta-analysis. In contrast, the optimal management for borderline resectable tumors is still debated. Compared with resectable PDA, borderline tumor is characterized by a higher risk of positive-margin resection with a subsequent higher risk of recurrence[34]. Although the prognosis of borderline resectable patients is significantly better than that of LAPC, survival rates are worse than those of resectable tumors[39]. Moreover, the role of arterial resection (AR) during pancreatectomy in borderline tumors has been analyzed in a recent systematic review published by

Mollberg *et al*[40]. Perioperative morbidity rates of patients with AR ranged from 17% to 100% (median 53.6%) with a median mortality rate of 12% (range: 0%-45.5%) compared to 2.6% in standard pancreatic resection[29,30]. Pancreatectomy with AR then increases the risk of mortality fivefold, without significant advantages in terms of long-term survival. These results demonstrate that the artery involvement by PDA, implies a more aggressive tumor biology, and these neoplasms should be considered as locally advanced despite the feasibility of surgical resection. Also, the involvement of the splenic artery has been demonstrated to be an adverse prognostic factor in body/tail PDA[41]. Neoadjuvant therapy is specifically beneficial in borderline resectable tumors and increases the fraction of resectable tumors. Katz *et al*[42] reported that 78% of patients completed neoadjuvant therapy and restaging, and 41% of them eventually underwent pancreatectomy. In this light, they suggest that neoadjuvant treatment could be considered to select properly patients who can benefit from surgery.

FURTHER DIAGNOSTIC TOOLS TO ASSESS RESECTABILITY

In several cases of patients with seemingly resectable tumors, clinical and radiological work-up could be lacking and further examinations are warranted in order to clarify doubtful findings (*i.e.*, elevated CA19.9 or persistence of abdominal pain). It has been observed that, in about 15% of patients with radiologically resectable PDA, surgery does not improve survival[43]. These patients are at high risk of early death despite radical surgery and they should be identified preoperatively using additional tests. Endoscopic ultrasound (EUS) is complementary to CT in the staging of the disease and in the detection of vascular invasion (SMA, SMV, and celiac axis) and lymph node metastasis[44,45]. Also EUS with fine needle aspiration (FNA) is preferable to CT-guided FNA in patients with resectable disease because of better diagnostic yield, safety, and potentially lower risk of peritoneal seeding[30]. EUS could be also helpful for obtaining a cytological grading of the tumor preoperatively. Among patients with borderline resectable PDA, the presence of a poorly differentiated or anaplastic tumor is another factor that shifts the management toward neoadjuvant treatment[6]. Nevertheless, the accuracy of FNA in the assessment of tumor grading has not been validated so far. Diagnostic staging laparoscopy to rule out metastasis not visible at standard imaging is routinely used in some institutions prior to surgery or chemoradiation or in patients with high risk for disseminated disease. Selective use of laparoscopy may be more appropriate and will probably be a more cost-effective approach[46]. The role of positron emission tomography (PET) with [18]fluorodeoxyglucose is still unclear, although it may be considered after formal pancreatic CT protocol in patients with high risk of metastasis, but it is not a substitute for high-quality, contrast-enhanced CT[30]. Nowadays, PET-CT favorably alters management more often when used for therapeutic monitoring compared to staging or restaging[47].

Beyond these imaging techniques, genetic status of a pancreatic carcinoma can be used to predict widespread metastatic failure. Several studies have demonstrated that there are different genomic alterations in PDA[48,49]. The most important are point mutations of *KRAS, CDKN2A/ p16*, *TP53*, amd *SMAD4/DPC4*. Yonezawa *et al*[50] have analyzed the genetic abnormalities in precursor lesions such as pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasms, mucinous cystic neoplasms and their relation to PDA. They have found that KRAS mutation in PDA is 75%-100%, and SMAD4/ DPC4 inactivation is seen in 55% of PDA patients. The low expression levels of SMAD4 are associated with an high rate of lymph node metastasis and poor survival[49]. Tanaka *et al*[51] have reported that loss of SMAD4 protein expression and chromosome 18q deletion were distinctly associated with metastasis. Determinations of DPC4 status at initial diagnosis may be of value in stratifying patients into treatment regimens related to local control *vs* systemic therapy[52]. Locally advanced carcinomas from patients with no documented metastatic disease uncommonly showed loss of DPC4 expression (22%) as compared with carcinomas from patients with extensive metastatic burden in which the rates of DPC4 loss approached 75%. In this setting, patients with DPC4-positive carcinomas would receive greater clinical benefit from intensive local control by CRT compared to patients with DPC4-negative carcinomas in which systemic chemotherapy alone may be more appropriate[53]. The advantage of SMAD4/DPC4 expression as a prognostic indicator is that it is potentially assessable preoperatively or during staging laparoscopy, whereas other factors, such as margins, perineural invasion and lymph node status are determined only after resection.

CONCLUSION

Surgical resection is still the only curative treatment for PDA. Oncological treatments have improved survival in patients with pancreatic cancer, also by increasing the rate of down staging and consequently of resectability. This improvement is probably also due to better patient selection by pancreatic surgeons. Nevertheless, current definitions of resectable, borderline resectable and locally advanced tumors are based only on radiological parameters and do not take into consideration the biology of the disease. Indeed, in borderline resectable disease a clear advantage in terms of survival has not been demonstrated for up-front surgery. Furthermore, surgery for borderline resectable is burdened by a high rate of morbidity and mortality that does not improve survival. In this light, a new concept of borderline pancreatic cancer has to include clinical and biological aspects (type and duration of symptoms, CA19.9 level, and immunohistochemistry). The selection of patients who will benefit from surgery has to be improved in the setting of an MDT discussion

that also considers further examinations.

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2014 ADVANCES IN PANCREATIC CANCER

Pancreatic cancer and its stroma: A conspiracy theory

Zhihong Xu, Srinivasa P Pothula, Jeremy S Wilson, Minoti V Apte

Zhihong Xu, Srinivasa P Pothula, Jeremy S Wilson, Minoti V Apte, Pancreatic Research Group, South Western Sydney Clinical School, Faculty of Medicine, The University of New South Wales, Sydney, NSW 2170, Australia

Zhihong Xu, Srinivasa P Pothula, Jeremy S Wilson, Minoti V Apte, Ingham Institute for Applied Medical Research, Sydney, NSW 2170, Australia

Author contributions: Xu Z and Apte MV wrote the paper; Xu Z, Pothula SP, Wilson JS and Apte MV revised the paper.

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Correspondence to: Minoti V Apte, Professor, Pancreatic Research Group, South Western Sydney Clinical School, Faculty of Medicine, The University of New South Wales, Liverpool, Sydney, NSW 2170, Australia. m.apte@unsw.edu.au

Telephone: +61-2-87389029 Fax: +61-2-96029441

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Abstract

Pancreatic cancer is characterised by a prominent desmoplastic/stromal reaction that has received little attention until recent times. Given that treatments focusing on pancreatic cancer cells alone have failed to significantly improve patient outcome over many decades, research efforts have now moved to understanding the pathophysiology of the stromal reaction and its role in cancer progression. In this regard, our Group was the first to identify the cells (pancreatic stellate cells, PSCs) that produced the collagenous stroma of pancreatic cancer and to demonstrate that these cells interacted closely with cancer cells to facilitate local tumour growth and distant metastasis. Evidence is accumulating to indicate that stromal PSCs may also mediate angiogenesis, immune evasion and the well known resistance of pancreatic cancer to chemotherapy and radiotherapy. This review will summarise current knowledge regarding the critical role of pancreatic stellate cells and the stroma in pancreatic cancer biology and the therapeutic approaches being developed to target the stroma in a bid to improve the outcome of this devastating disease.

Core tip: This review summarises current knowledge about the role of pancreatic stellate cells in production of cancer stroma, the mechanisms mediating stromal-tumour interactions and novel therapeutic approaches developed on the basis of our increasing understanding of the critical influence of stromal elements on disease progression.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in developed countries[1,2]. From 1999 to 2008, the incidence rate of pancreatic cancer increased nearly 1% in United States, although the reason for this increase is unknown[3]. In general, pancreatic cancer refers to pancreatic ductal adenocarcinoma (PDAC) that accounts for around 90% of pancreatic cancer[4]. Since the clinical symptoms can be vague, patients are often diagnosed late, with regional invasion or distant metastasis already evident at first consultation[5-7]. The overall five-year survival rate of pancreatic cancer is approximately 6% in the United States[2], less than 6% across Europe[8] and 5% in Australia[9]. Despite the concerted endeavours
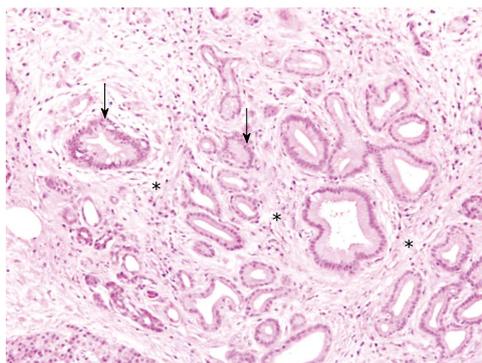
Figure 1 Pancreatic cancer and stromal reaction. A representative HE stained human pancreatic cancer tissue section showing duct-like and tubular structures (malignant elements, examples highlighted by arrows) infiltrating into and embedded in a highly fibrotic stromal reaction (examples highlighted by asterisks).

of clinicians and scientists over several decades, pancreatic cancer remains a devastating disease with a poor outcome.

Risk factors for pancreatic cancer include age, smoking, race, diabetes and chronic pancreatitis, the last being the strongest known risk factor for this disease. Patients with a more than 5 year history of chronic pancreatitis have a greater than 14-fold risk of developing pancreatic cancer compared to the general population[10,11]. 40% of hereditary pancreatitis (a form of chronic pancreatitis) patients are likely to develop pancreatic cancer[12-14], while patients with tropical pancreatitis have been reported to have a 100-fold increased risk and an earlier onset of the disease compared to sporadic cases[15,16]. The mechanisms underlying the increased propensity for patients with chronic pancreatitis to develop pancreatic cancer are not fully elucidated. Recent studies suggest several signalling pathways known to be active in inflammatory disease, may be involved in the progression from pancreatitis to pancreatic cancer.

One such signalling molecule known to play a key role in inflammation is the transcription factor, nuclear factor κB (NF-κB). Activation of NF-κB leads to the release of several proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and induces anti-apoptotic responses *via* Bcl-xL[17]. In addition to its observed activation in pancreatitis, NF-κB activity has also been observed in pancreatic cancer tissue. It has been shown to modulate angiogenesis *via* vascular endothelial growth factor (VEGF) and urokinase, and apoptosis possibly *via* antiapoptotic proteins such as Bcl-xL, cIAP1 (inhibitor of apoptosis protein), cIAP2, TRAF1 (TNF receptor-associated factor) and TRAF2[10,18]. NF-κB also negatively regulates the expression of p53, which is a tumour suppressor gene[19]. Further evidence for a role of NF-κB in cancer comes from an *in vivo* study using a NF-κB inhibitor (LC-1) in a xenograft pancreatic cancer mouse model. This inhibitor was found to reduce tumour growth and was associated with decreased ex-

pression of cyclin D1, a protein required in cell cycle G1/S transition[18,20].

K-Ras is another signalling pathway that is involved in both chronic pancreatitis and pancreatic cancer. *K-Ras* mutations exist in about a third of chronic pancreatitis patients[21]. Daniluk *et al*[22] reported that oncogenic K-Ras activation by inflammation in the mouse pancreas promoted development of chronic pancreatitis and pancreatic cancer precursor lesions. In another study, mutant K-Ras in acinar cells resulted in neoplastic lesions in mouse pancreas that progressed to pancreatic cancer in conjunction with *p53* deletion[23]. Logsdon *et al*[24] have postulated that Ras activity is the direct link between chronic pancreatitis and pancreatic cancer. The induction of chronic pancreatitis in a genetically engineered mouse model with K-Ras overexpression led to the development of primary pancreatic tumours as well as metastasis[25-28]. Collins *et al*[29] have shown in mice bearing inducible *K-Ras* mutations, that oncogenic K-Ras initiates pancreatic carcinogenesis by hindering pancreatic repair after caerulein-induced pancreatitis. Importantly, inactivation of *K-Ras* mutation in these mice leads to tumour regression suggesting a role for oncogenic K-Ras in the maintenance of pancreatic cancer.

In addition to *K-Ras* mutations, a number of genetic mutations are frequently reported in pancreatic cancer. Biankin *et al*[30] performed exome sequencing and copy number analysis in a cohort of 142 sporadic PDAC cases and reported multiple significantly mutated genes, including the known mutations - *KRAS*, *TP53*, *CDKN2A*, *SMAD4*, *MLL3*, *TGFBR2*, *ARID1A*, *SF3B1* and importantly, previously unidentified mutations such as *EPC1*, *ARID2* (chromatin modifications), *ATM* (DNA damage repair), *ZIM2* (transcription regulation), *MAP2K4* (Toll-like receptor signalling pathway), *NALCN* (sodium channel activity), *SLC16A4* (monocarboxylate transporter), *MAGEA6* (protein binding). The accumulation of genetic mutations leads to the development of precursor lesions, the most common of which are pancreatic intraepithelial neoplasia (PanIN)[10,31,32]. PanINs are normally found in smaller diameter pancreatic ducts, with the microscopic features progressing from PanIN-1A to PanIN-3 and finally to overt PDAC.

Histopathologically, PDAC is characterised by duct-like and tubular structures (malignant elements) infiltrating into and embedded in a highly fibrotic stromal reaction[5,33] (Figure 1). This stromal reaction is comprised of abundant extracellular matrix (ECM), stromal cells, blood vessels/endothelial cells, immune cells, nerves/neurons and other soluble proteins, *e.g.*, cytokines, growth factors[10].

The ECM itself is composed of proteins such as type I collagen, fibronectin and laminin as well as proteoglycans, such as hyaluronan, which is a non-sulphated glycosaminoglycan secreted by cancer cells. Hyaluronan is known to bind to CD44 (its receptor) and to influence angiogenesis, epithelial-mesenchymal transition (EMT) and chemo-resistance, possibly *via* the regulation of receptor tyrosine kinase and small GTPase[34].
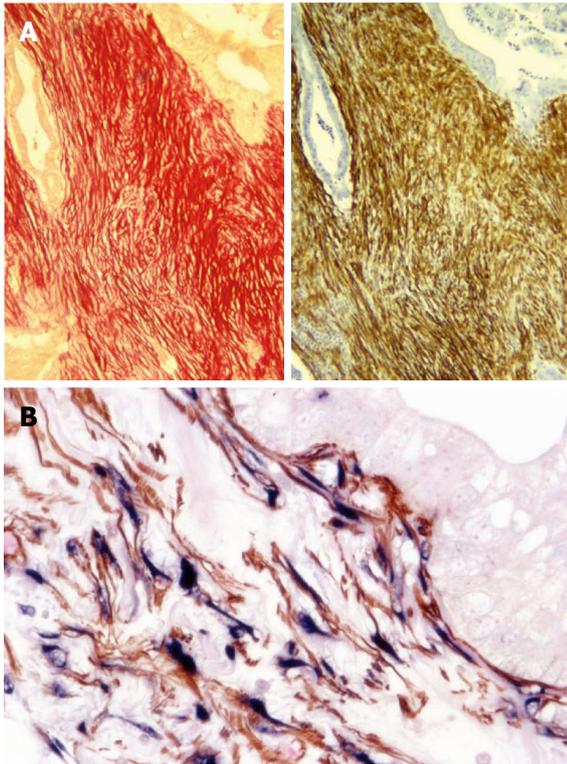
Figure 2 Pancreatic stellate cells are the source of collagen in stroma.
A: A representative pair of serial sections of human pancreatic cancer tissue shows that Sirius Red staining for collagen (red) co-localises with immunohisto-chemical staining for α-smooth muscle actin (α-SMA) (brown), suggesting the presence of activated pancreatic stellate cells (PSCs) in the stroma of pancreatic cancer[33]. Reprinted with permission from *Wolters Kluwer Health* (Apte *et al*[33]); B: Immunohistochemistry for α-SMA (brown) and in situ hybridisation for procollagen α1 mRNA (blue), reveals colocalisation of α-SMA and procollagen mRNA on human pancreatic cancer tissue indicating that active PSCs are the major source of collagen in tumour stroma. Reprinted with permission from *Elsevier* (Apte *et al*[43]).

Collagen I promotes pancreatic cancer cell adhesion, proliferation and migration *via* integrin α2β1[35]. Collagen, fibronectin and laminin are also found to be associated with increased chemo-resistance of pancreatic cancer cells *in vitro*[36].

There is now unequivocal evidence that fibrosis of the pancreas is produced by pancreatic stellate cells (PSCs)[33]. PSCs were first isolated from rat pancreas in 1998 by Apte *et al*[37] using a density centrifugation method. A similar method to isolate human PSCs from histologically normal human pancreas was later described by the same group[38]. Bachem *et al*[39,40] reported isolation of human PSCs from fibrotic pancreatic tissue of patients with chronic pancreatitis[39] and pancreatic cancer[40] using an explant technique. With the availability of these methods to isolate and culture of PSCs, researchers have been able to make significant advances in the understanding PSC biology.

PSCs are resident cells of the pancreas and comprise about 4%-7% of total parenchymal cells in the gland[37,41]. There are abundant vitamin A containing lipid droplets in the cytoplasm, which is a marker of quiescent PSCs. PSCs synthesise the ECM proteins collagen, fibronectin and laminin. They also express the matrix metallopro-

teinases (MMPs), MMP2, 9 and 13 that degrade ECM and the tissue inhibitors of metalloproteinases (TIMPs), TIMP 1 and 2 that inhibit the activity of MMPs. Therefore, PSCs are thought to play an important role in maintaining a balance between ECM synthesis and degradation to maintain normal pancreatic architecture in health[41]. During pancreatic injury, PSCs are activated by factors such as ethanol (a known cause of chronic pancreatitis) and its metabolites, bacterial endotoxin, oxidant stress, cytokines and growth factors. Activated PSCs lose vitamin A droplets, assume a myofibroblast-like phenotype, express the cytoskeletal protein α-smooth muscle actin (α-SMA) and synthesise excessive amount of ECM proteins leading to fibrosis[37,41-44].

In a bid to shed some light on the differences between stellate cells in health and diseases, we have conducted microarray studies to examine differences in gene expression in human PSCs obtained from normal pancreas (benign pancreatic diseases: serous cystadenoma, *etc.*) *vs* the disease-activated PSCs isolated from chronic pancreatitis and pancreatic cancer tissue[45]. Multiple genes were found to be differentially expressed. Validation studies confirmed that MMP3 was upregulated 32.25 fold, collagen type IVα1 (a basement membrane component) was downregulated 2.25 fold and syndecan-2 (a transmembrane heparan sulphate proteoglycan that plays a role in cell binding, cytoskeletal organization, migration and invasion[46]) was downregulated 2.04 fold. These three genes are postulated to be involved in ECM remodeling function and motility of PSCs. However, in depth characterisation of the role of these genes in the functional modulation of PSCs remains to be undertaken.

IDENTIFICATION OF PSCS AS SOURCE OF ECM DEPOSITION IN STROMA

Up until just under a decade ago, the prominent stroma/ fibrosis in pancreatic cancer had been largely ignored. In 2004, Apte *et al*[33] demonstrated that PSCs produced the collagenous stroma in pancreatic cancer. Using serial sections of human pancreatic cancer tissue, the authors showed that the PSC activation marker α-SMA, co-localised with Sirius red stain for collagen (Figure 2A), as well as with PSC selective markers, desmin and glial fibrillary acidic protein. Most importantly, co-localization of staining for α-SMA and procollagen mRNA (using in situ hybridization) indicated that activated PSCs were the predominant source of the collagen in stroma (Figure 2B). The authors also found that conditioned medium from human pancreatic cancer cell lines increased the proliferation and activation of PSCs *in vitro*[33]. This was one of the first studies to initiate investigations into tumour stroma interactions in pancreatic cancer.

ROLE OF PSCS AS PROGENITOR CELLS

Recent evidence suggests that in addition to synthesising ECM proteins, PSCs may have other roles within the pan-

Pancreatic cancer cells
↑Proliferation
↓Apoptosis ⎤
         ⎦ ↑Survival
↑Migration/invasion
↑Metastasis
↑Stem cell niche

Activated pancreatic stellate cells
↑Proliferation
↑Fibrosis/ECM synthesis
↑Angiogenic factors, MMPs
↑Migration and metastasis
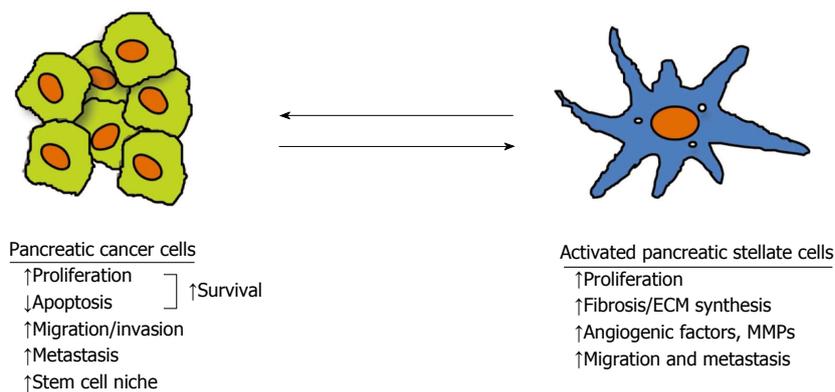
Figure 3 Interaction between pancreatic cancer cells and pancreatic stellate cells. Pancreatic cancer cells stimulate the proliferation, extracellular matrix (ECM) production, angiogenic factors and matrix metalloproteinase (MMP) expression, as well as migration of pancreatic stellate cells (PSCs); conversely PSCs increase proliferation and reduce apoptosis leading to increased survival, increase cancer cell migration and facilitate a cancer stem cell niche. The overall effect of interaction between pancreatic cancer cells and PSCs facilitates cancer progression[43].

creas, for example, as progenitor cells. In this regard, Mato *et al*[47] isolated and expanded pancreatic cells from lactating rats using mitoxantrone (a drug that acts through multidrug transporter systems) selection. They have reported that the surviving, mitoxantrone-resistant cells showed a PSC-like morphology (fibroblast-like with vitamin A lipid droplets), expressed the stem cell marker ABCG2 transporter (ATP binding cassette G2 transporter) and were able to secrete insulin after cell differentiation. More intriguingly, a recent study by Kordes *et al*[48] has reported that clonally expanded rat PSCs, when injected into hepatectomised recipient rats, are able to migrate to the liver and to reconstitute large parts of the liver by differentiating into hepatocytes and cholangiocytes, whereas muscle fibroblast do not show any such transformations.

IN VITRO INTERACTION BETWEEN PANCREATIC CANCER CELLS AND PSCS

In vitro studies assessing interactions between pancreatic cancer cells and PSCs usually involve co-culture experiments with these two types of cells (from humans and rodents) and/or exposure of one type of cell to the conditioned medium from the other. Pancreatic cancer cells have been shown to increase the proliferation and migration of PSCs as well as to induce the synthesis of collagen type I and fibronectin by PSCs[33,40,49]. Studies using neutralising antibodies have indicated that cancer cell-induced PSC proliferation and migration is mediated by platelet derived growth factor (PDGF), while the increase in synthesis of collagen I and fibronectin is modulated by the pro-fibrogenic factors, basic fibroblast growth factor (FGF-2) and TGF-β1 from cancer cells[33,40,49].

Other recently identified factors that may play a role in the interaction between cancer cells and PSCs include: (1) Cyclooxygenase (COX)-2, this enzyme is known to be constitutively expressed by PSCs. It catalyses reactions that transform arachidonic acid to prostaglandin H₂, the latter is then converted into prostaglandins, prostacyclin

and thromboxanes to modulate inflammation, immune responses, mitogenesis, *etc*.[50]. Conditioned medium from cancer cells increases COX-2 expression and proliferation of PSCs. Mitogen-activated protein kinase kinase inhibitor, U0126, was shown to inhibit the cancer cell-induced increase in COX-2 expression in PSCs, while a COX-2 inhibitor, NS398, prevented cancer cell-stimulated PSCs proliferation, suggesting a role for COX-2 in cancer cell - PSC interactions[51]; and (2) Trefoil factors (TFF), a family of proteins secreted by the gastrointestinal mucosa, play a role in restitution after mucosal damage[52,53]. TFF1 expression is elevated in PDAC tissue and is detected in the majority of pancreatic cancer cell lines. It stimulates PSC proliferation and migration, as well as cancer cell invasion. The receptor and downstream signalling pathway for TFF1 are yet to be identified. TFF2 is expressed in chronic pancreatitis and PDAC, and has been shown to stimulate cancer cell migration in transwell membrane or wound healing experimental settings[53]. TFF2 is postulated to act *via* the chemokine receptor type 4 (CXCR4, a receptor for stromal derived factor-1) that is also expressed by pancreatic cancer cell lines and PSCs. Interestingly, CXCR4 expression is elevated in PanINs and in PDAC and promotes cancer cell metastasis, growth and survival[52,54].

In parallel with the above described studies examining the influence of cancer cells on PSCs, researchers have been studying the effects of PSCs on cancer cells (Figure 3). Conditioned medium from PSCs has been shown to stimulate pancreatic cancer cell proliferation, and this effect is inhibited by pretreatment of the medium with a PDGF neutralising antibody. Since cancer cells express PDGF receptor, it is thought that PDGF in PSC conditioned medium mediates the observed effect[49]. Conditioned medium from PSCs also stimulates cancer cell migration, invasion and colony formation (again mediated by PDGF), but inhibits apoptosis, and increases resistance to chemotherapy and radiation. ERK1/2 and Akt kinases in cancer cells are known to increase after incubation with PSC conditioned medium[49,55], suggesting

that these pathways mediate the responses of cancer cells to PSC conditioned medium.

PSCs secrete a cell adhesion protein named periostin, which has been found to stimulate cancer cell growth and to confer resistance on the latter to serum starvation and hypoxia. Periostin may also act in an autocrine manner on PSCs themselves leading to increased collagen I and fibronectin production. Collagen I might subsequently perpetuate the PSCs activation. Notably, cancer cells have been shown to induce periostin expression, and modulate collagen I and fibronectin expression in PSCs thus creating a supportive microenvironment for the tumour[56].

Evidence is now accumulating to indicate that PSCs may also influence EMT in cancer cells. A recent study has reported that a subpopulation of pancreatic cancer cells express endoglin (CD105, a TGF-β co-receptor); upon co-culture with PSCs, the proportion of CD105 positive cancer cells increases, and these cells exhibit a greater increase in migration activity compared to CD105 negative cells. Interestingly, in the CD105 positive population of cancer cells, mRNA expression of E-cadherin (an epithelial cell marker) is suppressed while vimentin (a mesenchymal cell marker) is over expressed, indicating that CD105 expression is associated with EMT in cancer cells[57]. These results suggest that PSCs may induce EMT in pancreatic cancer cells. This concept is supported by another study. Using organotypic *in vitro* cultures (collagen I coated culture wells), Froeling *et al*[58] reported that co-culture of cancer cells and immortalised human PSCs (obtained from donor pancreas and transfected with retroviruses containing cDNA encoding human telomerase reverse transcriptase) in this system resulted in decreased E-cadherin expression and increased beta-catenin expression of cancer cells, again signifying a transformation of the cells to a more mesenchymal phenotype.

While, as described above, there is strong evidence that PSCs significantly influence cancer cell function, some doubts have been raised in a recent study as to whether all PSCs uniformly exert such effects. Ikenaga *et al*[59] have demonstrated the existence of functional heterogeneity among the PSC population. They have reported that a sub-population of PSCs which express CD10, a cell membrane associated MMP, exerts a more inductive effect on cancer cell invasion and proliferation than CD10 negative PSCs. Thus, this study suggests that human PSCs (isolated from pancreatic tissue of pancreatic cancer patients) may differ in their ability to affect cancer cells, and explain, at least in part, the heterogeneity observed in patients with regard to rate of disease progression.

Recurrence is a well recognised feature of pancreatic cancer and recent studies suggest that this may be related to a population of cancer stem cells (identified by expression of markers such as CD24, CD44 and CD133) that are resistant to treatment[43,57]. Interestingly, PSCs have been reported to increase the stem cell characteristics of cancer cells by inducing the expression of cancer stem cell-related genes ABCG2, Nestin and LIN28[60]. This surviving cancer stem cell niche may be an important factor in pancreatic cancer recurrence.

IN VIVO INTERACTION BETWEEN PANCREATIC CANCER CELLS AND PSCS

While the *in vitro* studies noted above provided robust data on the direct interaction between cancer cells and PSCs, *in vivo* studies are essential in terms of biological/whole organism relevance. In this regard, two earlier clinical studies have reported findings to support a role for the stroma in cancer progression. Watanabe *et al*[61] have reported that the presence of fibrotic foci (which the authors postulated as representing intratumoural fibroblast proliferation) was associated with shorter survival in advanced pancreatic cancer, while Erkan *et al*[62] have reported that high α-SMA/collagen ratios in tumours correlated with poor prognosis. However, as detailed below, most of the *in vivo* evidence in support of tumour-stromal interactions in pancreatic cancer comes from experimental studies using tumour xenografts and genetically engineered mouse models.

Using a subcutaneous mouse model of pancreatic cancer, wherein tumours were produced by injecting a suspension of pancreatic cancer cells, alone or in combination with PSCs, into the flanks of mice, Bachem *et al*[40] showed that mice injected with both cell types exhibited larger tumours than those injected with cancer cells alone. Histological assessment of tumours indicated that cancer cell proliferation and stromal content were both increased in the presence of PSCs, an effect that would contribute to the observed increase in tumour volume. However, subcutaneous xenograft models have obvious limitations, since they do not replicate the appropriate microenvironment, nor can they provide information on metastasis.

In 2008, two research groups used similar approaches involving injection of a mixture of pancreatic cancer cells (cell lines MiaPaCa-2 or BxPC-3) and human PSCs (either primary culture[49] or immortalised cells[55]) into mouse pancreas. The results from these studies demonstrated that co-injection of cancer cells and PSCs yielded larger tumours with higher cancer cell density revealed by cytokeratin staining, increased fibrosis as determined by Masson's trichrome staining and higher number of activated PSCs (increased α-SMA staining). The incidence of metastasis was also higher in the presence of PSCs in both studies. It is interesting to note that these facilitatory effects of PSCs on cancer progression are not restricted to the PSCs derived from resected pancreatic cancer tissue (*i.e.*, PSCs that have been exposed to cancer cells prior to isolation). Xu *et al*[63] have demonstrated that normal human PSCs (isolated from normal pancreas) exert a similar facilitatory effect on tumour growth and metastasis in an orthotopic mouse model of pancreatic cancer. These findings suggest that PSCs are relatively quick to acquire tumour inductive properties after a relatively short exposure to cancer cells, supporting the concept that cancer cells are highly efficient and effective at recruiting surrounding PSCs, so as to set up a conducive microenvironment (such as ECM) for their own growth. Indeed, direct effect on cancer cells of ECM (produced by PSCs) have been demonstrated by Scaife *et al*[64]. The authors assessed

Mesentery

Liver   Diaphragm
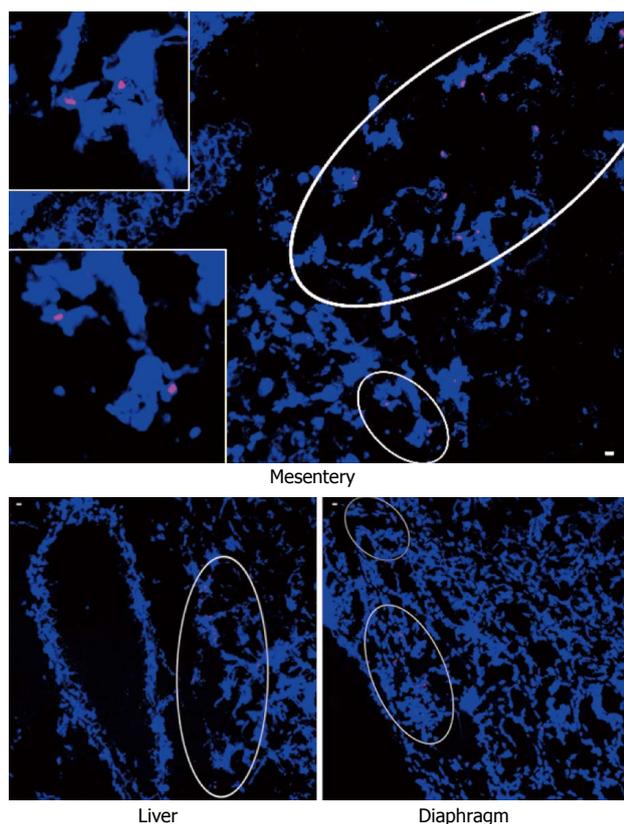
Figure 4 Identification of human pancreatic stellate cells from primary tumour in metastatic nodules. A representative photomicrograph showing Y chromosome positive cells in metastatic nodules in the mesentery (inserts are high power views of the circled regions), liver and diaphragm from mice (female) injected with female pancreatic cancer cells + male human pancreatic stellate cells, using fluorescent *in situ* hybridisation for the Y chromosome. Reprinted with permission from *Elsevier* (Xu et al[63]).

cancer progression in an orthotopic mouse model of pancreatic cancer by implanting a mixture of cancer cells and synthetic ECM (a hyaluronan-based hydrogel) into the pancreas of nude mice; the encapsulation of cancer cells within ECM yielded larger tumours than cancer cells suspended in serum-free media.

In contrast to orthotopic models where tumours are produced in immunocompromised mice by xenografts of human pancreatic cancer cells and PSCs, genetically engineered mouse models exhibit the development of spontaneous pancreatic tumours with a prominent endogenously produced stromal reaction. These models include KPC mice (Kras$^{LSL-G12D/+}$; Trp53$^{LSL-R172H/+}$; Pdx$^{cre/+}$), KPGC mice (Kras$^{LSL-G12D/+}$; Trp53$^{LSL-R172H/+}$; R26$^{LSL-GFP/+}$; Pdx$^{cre/+}$), and TGFβ type II receptor organ specific knockout in the mouse pancreas (Kras$^{LSL-G12D/+}$; TGFβr2^{floxflox}; Ptf1a$^{cre/+}$). The tumours develop spontaneously with lesions progressing from preinvasive ductal changes to overt carcinoma and metastases. The stromal reaction also increases over time and importantly, activated PSCs are observed at the earliest time point (PanIN stages)[65-68]. It is anticipated that such models will be increasingly utilised to assess mechanisms of stromal-tumour interaction and new therapeutic strategies in pancreatic cancer.

ANGIOGENESIS IN PANCREATIC CANCER

Pancreatic cancer is poorly perfused, with the blood vessels in the tumours being often disorganised with irregular diameters, abnormal multiple branching, disrupted endothelial cells junctions and missing or disordered basement membrane. These changes cause the microvasculature of tumours to become leaky[69]. Angiogenesis is a complex process, and the response of endothelial cells in different parts of the tumour likely depends on the balance of the pro-angiogenic and anti-angiogenic factors within the surrounding microenvironment. It is possible that as activated PSCs lay down increasing fibrous stroma in central areas of the tumour, the blood vessels in that area are compressed, leading to insufficient perfusion and hypoxia. However, at the invading front of the tumour, where the collagenous stroma is significantly less dense, endothelial cell proliferation in response to activated PSC secretions can occur in a relatively unrestricted manner.

Masamune *et al*[70] reported that hypoxia induces migration, type I collagen expression, and VEGF production in PSCs. Interestingly, conditioned medium of hypoxia-treated PSCs induces endothelial cell proliferation, migration, and angiogenesis *in vitro*, possibly *via* VEGF. Xu *et al*[63] demonstrated that even when collected under normoxic conditions, conditioned medium from PSCs stimulated tube formation (a method of measuring angiogenesis *in vitro*) by human microvascular endothelial cells, and that this effect was again mediated by VEGF. Other factors produced by PSCs under hypoxic conditions include FGF-2, angiopoietin-1, periostin and hypoxia inducible factor-1[70,71], which may also promote angiogenesis.

ROLE OF PANCREATIC STELLATE CELLS IN PANCREATIC CANCER METASTASIS

Metastasis occurs early in pancreatic cancer, and has long been regarded as a feature solely exhibited by cancer cells. However, this concept has been challenged in recent time with studies indicating that untransformed epithelial cells[72] and mesenchymal cells[73] may also have the capacity to metastasise. It is now accepted that cells can travel to metastatic sites through the circulation either as single cells, or more likely, as a cluster of cells. We believe that metastatic cell clusters in pancreatic cancer could comprise both cancer cells and PSCs from the primary tumour. In this regard, Xu *et al*[63] used a gender mismatch approach (female pancreatic cancer cells plus male human PSCs injected into the pancreas of female mice) to demonstrate the presence of Y-chromosome positive cells in metastatic nodules (Figure 4). These results indicated that male PSCs from primary tumours were able to (1) intravasate into blood vessels; (2) be transported in the circulation; and (3) extravasate from blood vessels at metastatic sites. This possibility was supported by *in vitro* studies showing that PSCs can migrate through an endothelial cell monolayer *in vitro* and this transendothelial migration is up-regulated by PDGF from cancer cells.

In view of the above, we propose that PSCs that have travelled to the metastatic site perform a very important initial function at the metastatic sites, which is to facilitate seeding, survival and proliferation of the metastatic cancer cells at those sites. Also important is the likelihood that PSCs, *via* secretion of chemokines, subsequently recruit local stromal cells within the metastatic site, which further facilitates cancer cell growth.

IMMUNE CELLS, IMMUNE EVASION AND PSCS IN PANCREATIC CANCER

It is well established that pancreatic cancer tissue is infiltrated with immune cells, such as T cells, B cells, NK cells, neutrophils and macrophages[43,74,75]. Higher levels of CD8^{+} T cell infiltration are correlated with a better survival[74,75], while macrophage and neutrophil infiltration show a negative correlation with survival[75]. Most recently, Ene-Obong *et al*[74] have reported that activated PSCs reduce the migration of CD8 positive T cell to cancer cells in human PDAC and the KPC mouse model of pancreatic cancer, indicating that PSCs may negatively modulate immune responses.

Immune evasion is also a characteristic of pancreatic cancer. Cancer cells have been shown to evade the host immune system by producing granulocte-macrophage colony-stimulating factor to suppress anti-tumour T cell immunity[76]. Recent studies suggest that PSCs may play a role in this process as well. PSCs in the stroma of PanIN lesions and around cancer cells produce galectin-1, a β-galactoside-binding protein[77,78], that binds to N-acetyllactosamine on membrane glycoproteins and induces apoptosis in T cells thus suppressing the immune response[79,80]. Fibroblast activation protein-α (FAP-α) known to be expressed by stromal cells, is another protein that has been reported to disrupt anti-tumour immunity. Depletion of the cells expressing FAP-α enabled immune response-associated tumour regression, supporting the notion that FAP-α might act as an immune suppressor in pancreatic cancer[81].

PANCREATIC STELLATE CELL AND ITS ROLE IN THE INITIATION OF NEOPLASIA

Evidence from recent studies is accumulating to indicate that PSCs might be activated at the earliest stages of carcinogenesis. Pandol *et al*[11] have found a distinct stromal reaction around PanIN lesions in a mouse model overexpressing Kras[G12D] that leads to pancreatic carcinogenesis. This stromal reaction is characterised by extensive collagen deposition and abundant α-SMA staining indicating the presence of activated PSCs around PanIN lesions. These findings corroborate those reported earlier by Fukushima *et al*[82] showing that periostin (solely expressed by PSCs) was observed in intraductal papillary mucinous neoplasms of the human pancreas. To assess the interaction between early neoplastic cells and PSCs, Pandol *et al*[11] isolated PanIN cells from the Kras[G12D] mice, and exposed PSCs

to PanIN cell secretions. PSCs responded by increased proliferation, activation (α-SMA), fibronectin synthesis and MMP expression, indicating that preneoplastic cells have the capacity to activate PSCs in the early stages of carcinogenesis.

It is possible that in turn, these activated PSCs influence further progression of early lesions to established PDAC. In this regard, Funahashi *et al*[83] have reported that, nimesulide, a selective inhibitor of COX-2 (which as noted earlier is expressed by PSCs and implicated in PSC-cancer interactions[51]), retarded the progression of pancreatic cancer precursor lesions in a genetically engineered mouse model.

TARGETING THE STROMA OF PANCREATIC CANCER

The selection of treatment for pancreatic cancer patients depends on the stage of disease. The available options are surgery, chemotherapy, radiotherapy and recently developed targeted therapy, such as growth factor inhibition. Chemotherapy is the most frequently used treatment option for pancreatic cancer patients at different stages. Surgical resection with curative intent is only suitable for less than 20% of patients who have localised and early stage of pancreatic cancer[2,84]. Local recurrence is frequent and neoadjuvant and/or adjuvant therapies (chemotherapy and/or radiotherapy) are often required. For some advanced pancreatic cancers, surgery may be chosen to relieve obstruction and to improve the quality of life.

Gemcitabine was established as a first line chemotherapeutic drug for pancreatic cancer more than a decade ago, but it extends median overall survival only by several months[6]. Various combinations of chemotherapeutics have also been tried but regrettably the improvement has been negligible.

Based on an understanding of cancer cell biology and results from preclinical studies, several modalities targeting growth factor receptors and downstream signalling pathways have also been trialed. Unfortunately, these have not proved to be very successful. For example, the combination of erlotinib (an inhibitor of EGFR) and gemcitabine was shown to extend patient life by a mere two weeks *vs* gemcitabine alone. Other clinical trials involved inhibition of EGFR, VEGF and farnesyl-transferase by cetuximab, bevacizumab and tipifarnib respectively, but were not able to produce positive results[84]. The failure of translation of preclinical efficacy to the clinical situation may reflect the fact that many of the preclinical models used in these studies did not resemble human pancreatic cancer, in that, they lacked the stromal component.

In view of the above, it is clear that a comprehensive approach is needed to improve pancreatic cancer therapeutic efficacy. Given the increasingly recognised role of the stroma in cancer progression, there is a need to target not only cancer cells themselves but also the stromal elements in the tumour. The approaches discussed below have been built upon knowledge gained regarding PSC

biology and ECM composition.

The Hedgehog signalling pathway is thought to play an important role in PSC activation[85]. This pathway is crucial to embryonic development, and stem cell regulation in adults, but has also been implicated in tumour development. There are three Hedgehog ligands in mammals: Sonic, Indian and Desert Hedgehog[86]. This signalling pathway is inactive in health, and therefore not detectable in healthy adult pancreas[87]. In the absence of Hedgehog ligand, its cell membrane bound receptor, named Patched, represses another transmembrane protein, called Smoothened. The binding of Hedgehog ligand to Patched causes the repression of Smoothened to be lifted, leading to activation of downstream Gli proteins, a family of transcription factors that regulate genes related to cell functions such as cell differentiation, proliferation, apoptosis, adhesion and migration[67,88-90]. Abnormal activation of Hedgehog pathway has been shown in basal cell carcinoma, as well as lung, prostate, pancreatic cancer[89]; this activation can be Hedgehog ligand dependent (as in pancreatic cancer) or due to mutation of Patched (as in basal cell carcinoma)[91]. In pancreatic cancer, Smoothened was shown to be highly expressed by PSCs, and Sonic Hedgehog ligand to be expressed by pancreatic cancer cells only[88]. Feldmann *et al*[92] administered cyclopamine, a Smoothened antagonist, in a Pdx1-Cre;LsL-Kras^{G12D}; Ink4a/Arf$^{lox/lox}$ transgenic pancreatic cancer mouse model (crossbred LsL-Kras^{G12D}; Ink4a/Arf$^{lox/lox}$ and Pdx1-Cre; Ink4a/Arf$^{lox/lox}$) resulting in an extension of the overall median survival from 61 to 67 d. Olive *et al*[67] administered IPI-926, a semisynthetic derivative of cyclopamine, alone or in combination with gemcitabine in a KPC pancreatic cancer mouse model. IPI-926 binds to and inhibits Smoothened to keep Gli in an inactive form[91]. IPI-926 decreased collagen 1 content in stroma associated with a decrease in the proliferation of α-SMA positive stromal cells and transiently increased blood vessel density in primary tumours in KPC mice. The authors reported an improvement in delivery of chemotherapeutic agent to the tumours and an extension of the median survival from 11 to 25 d. Hwang *et al*[88] applied another Smoothened inhibitor AZD8542 in an orthotopic xenograft model of pancreatic cancer produced by a mixture of PSCs and cancer cells in different proportions (0:1, 1:1 or 3:1) and showed that AZD8542 significantly reduced tumour volume, lowered metastasis, decreased Hedgehog downstream signalling activity *via* decreased GLI 1 expression and increased tumour vascularity in tumours with a 3:1 proportion of PSCs to cancer cells. These studies imply that Sonic Hedgehog acts in a paracrine manner on stroma to facilitate pancreatic cancer progression, and that Hedgehog inhibition represents a potentially useful additional treatment approach for pancreatic cancer.

There are now several clinical trials targeting Sonic Hedgehog pathway inhibition in pancreatic cancer[6]. Unfortunately, despite encouraging results in phase I trials, the most recent phase Ⅱ trial of gemcitabine and IPI-926 has resulted in a disappointing outcome. The trial had to be halted due to progressive disease and decreased median overall survival in pancreatic cancer patients treated with IPI-926 and gemcitabine[6]. The reasons for the failure of this drug in the clinical setting are not entirely clear. The disappointing clinical outcome may reflect the fact that: (1) results from a single preclinical model are not sufficient to account for the heterogeneity of human pancreatic cancer; and (2) the effects described by Olive *et al*[67] in the preclinical model, particularly with regard to perfusion, were transient. Before taking treatments to the clinic, it would be prudent to ensure that robust, long lasting effects were demonstrable in the preclinical setting.

Most recently, researchers have utilised other compounds to target the stroma of pancreatic cancer. Kozono *et al*[93] administered pirfenidone (a pyridone compound that has been shown to be an effective antifibrotic agent in idiopathic pulmonary fibrosis) in subcutaneous and orthotopic models of pancreatic cancer. The results revealed that pirfenidone decreased the growth of tumours produced by the injection of a mixture of pancreatic cancer cells and PSCs, but not the growth of tumours produced by cancer cells alone. *In vitro*, the authors found that pirfenidone inhibited PSC proliferation, invasion and migration, and interrupted the interaction between pancreatic cancer cells and PSCs. These effects of pirfenidone were associated with decreased expression of PDGF-A, hepatocyte growth factor, periostin, collagen type I and fibronectin in PSCs, as well as reduced PSC activation as evidenced by decreased α-SMA expression in the cells. The findings suggest that pirfenidone regulates PSC function and inhibits cancer growth.

Angiotensin inhibitors, used routinely for treatment of hypertension, have been suggested as a potentially effective treatment in pancreatic cancer[94]. Angiotensin Ⅱ is known to be able to stimulate PSC proliferation, migration, ECM production, and increase expression of FGF, TGF-β and VEGF[95,96]. Thus, angiotensin inhibition is postulated to prevent the activation of PSCs. Masamune *et al*[96] administered an angiotensin Ⅱ type I receptor blocker, olmesartan, in a subcutaneous mouse model of pancreatic cancer. Similar to the effect of pirfenidone, olmesartan only inhibited the growth of tumours produced by injection of pancreatic cancer cells with PSCs, but not that of the tumours produced by cancer cells alone. The authors also reported that olmesartan reduced α-SMA expression and collagen deposition in tumours and decreased PSC proliferation and collagen I production *in vitro*. Similar to the results with olmesartan, Chauhan *et al*[97] have reported that another angiotensin Ⅱ receptor inhibitor, losartan, decreased the density of α-SMA positive cells, collagen and hyaluronan production in the stroma of pancreatic cancer in an orthotopic mouse model. The effects of losartan might be mediated though reduction of TGF-β1, connective tissue growth factor and endothelin-1 (downstream target of TGF-β1) expression, all of which regulate ECM production by PSCs. The authors reported that the reduction in stroma decreased the physical pressure within the tumour, leading to improved perfusion and more effective drug delivery. The studies
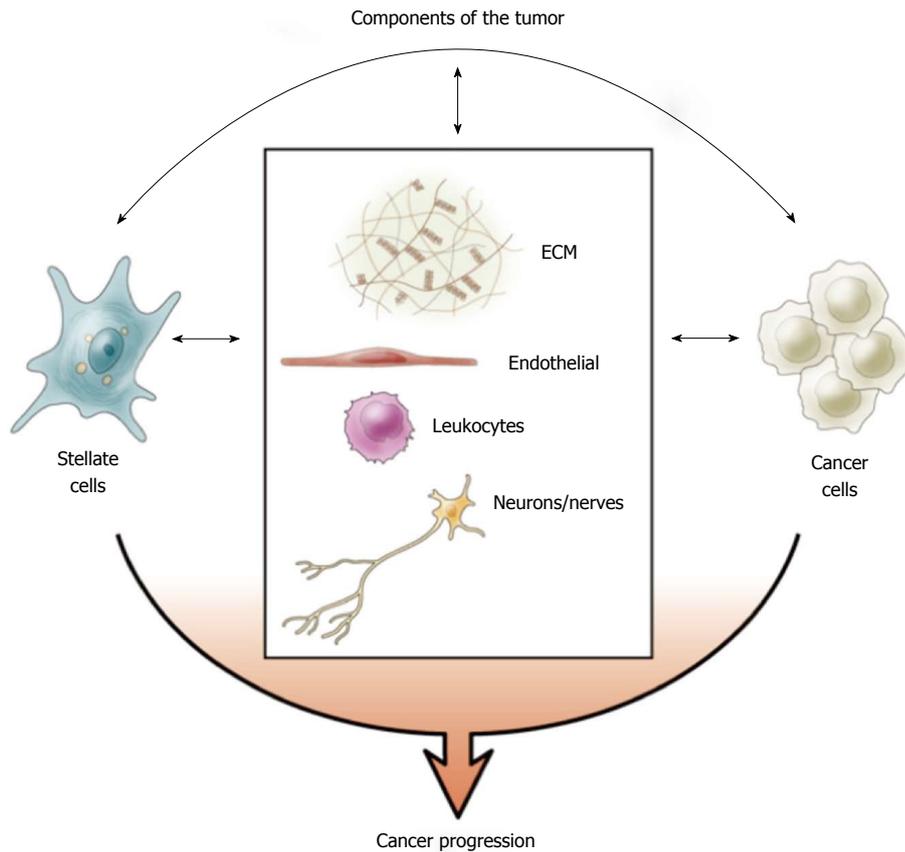
Figure 5 Tumour components. The stromal reaction of pancreatic ductal adenocarcinoma is comprised of pancreatic stellate cells (stromal cells), abundant extracellular matrix (ECM), blood vessels/endothelial cells, immune cells and nerves/neurons[43]. The interaction between cancer cells and the components of stroma facilitates cancer progression. Reprinted with permission from *Elsevier* (Apte *et al*[43]).

discussed above indicate that these compounds influence PSCs (stromal cells) function to inhibit tumour growth.

Taxanes [paclitaxel and docetaxel (semisynthetic analogue of paclitaxel)] have been widely used as chemotherapeutics for several cancers, such as breast, ovarian and non-small cell lung cancer. Paclitaxel inhibits the depolymerisation of microtubules in the cell and blocks cells in G2 and M phases resulting in cell death[98]. However, toxicity and insolubility in water of solvent-based paclitaxel significantly limit its clinical application. To overcome the toxicity of solvent, nab-paclitaxel was developed through homogenisation of paclitaxel and human serum albumin under high pressure to yield nano-particles about 130 nm in diameter. Paclitaxel is encapsulated by albumin in these nano-particles and becomes water soluble. As it is solvent free in comparison with parental compound, the toxicity of nab-paclitaxel is low and tolerated very well by pancreatic cancer patients. The albumin also enhances drug delivery through albumin facilitated receptor-mediated transcytosis[98].

Von Hoff *et al*[99] administered nab-paclitaxel alone or in combination with gemcitabine in a patient tumour-derived subcutaneous xenograft model. Nab-paclitaxel resulted in stromal depletion, increased the blood vessel diameter, increased expression of mNestin (an endothelial cell marker) in the tumour, and improved the delivery of gemcitabine. The mechanisms mediating these effects of nab-paclitaxel have not been fully elucidated. It has been observed that nab-paclitaxel accumulates in the proximity of tumour cells. Researchers have postulated that secreted protein acidic and rich in cysteine (SPARC), an albumin binding glycoprotein that is overexpressed in pancreatic cancer stroma[100], might contribute to the accumulation of nab-paclitaxel near tumour cells[98]. Analysis of SPARC expression in a clinical trial of gemcitabine and nab-paclitaxel combination has shown that high SPARC expression was correlated with significantly longer median overall survival compared to patients with low expression of SPARC[6]. Another mechanism that is also proposed to explain the synergistic effect of nab-paclitaxel and gemcitabine involves decreased metabolic inactivation of gemcitabine by cytidine deaminase which is destabilised by the increased production of reactive oxygen species in cancer cells following nab-paclitaxel administration[98]. The combination of nab-paclitaxel and gemcitabine is currently the subject of several ongoing clinical trials for locally advanced primary tumours and/or metastatic pancreatic cancer, as well as in neoadjuvant settings[6].

Two recent studies have directly targeted stromal ECM by using enzymes such as PEGylated human recombinant PH20 hyaluronidase (PEGPH20), to enzymatically degrade one of the predominant components of the ECM, hyaluronan. The authors reported that

PEGPH20 treatment led to stromal depletion, resulting in decompression of tumour vessels and an increase in tumour vascular patency without increasing vessel density. PEGPH20 also increased fenestrations in endothelia and interendothelial junction gaps that increased the permeability of the endothelium to macromolecules. Thus, the delivery of gemcitabine was improved with the PEGPH20 and gemcitabine combination significantly inhibiting tumour growth and extending the median survival of KPC mice from 15 to 28.5 d compared to gemcitabine alone in the study done by Jacobetz *et al*[34] or 55.5-91.5 d in the study done by Provenzano *et al*[68].

Researchers have recently also turned their attention to immune cells in pancreatic cancer stroma. CD40 is a member of the TNF receptor family and plays an important role in the development of anti-tumour T cell immunity. Beatty *et al*[101] performed studies on the KPC mouse pancreatic cancer model showing that the administration of CD40 agonist antibody activated macrophages, induced caspase-3 expression (an indicator of apoptosis) and decreased collagen I content in tumours. The treatment of CD40 agonist antibody alone or in combination with gemcitabine induced a similar rate (30%) of tumour regression. This regression appeared not to be related to $CD3^+$, $CD4^+$ and $CD8^+$ T cells in this *in vivo* study. The data from a clinical trial reported by the same group showed therapeutic efficacy of gemcitabine and CD40 agonist antibody on metastatic pancreatic cancer[101].

In summary, both *in vitro* and *in vivo* studies have clearly demonstrated a critical role of the stroma in the pathobiology of pancreatic cancer. PSCs interact closely with cancer cells to modulate cell proliferation, ECM production, migration and invasion of cancer cells. PSCs also play an important role in immune evasion, chemoresistance, angiogenesis and recurrence of pancreatic cancer (Figure 5). It is now increasingly clear that targeting tumour cells alone is insufficient to improve pancreatic cancer clinical outcome. Results from preclinical models and recent (albeit early) clinical trials provide vital evidence to support the concept that a comprehensive and combinatorial approach targeting both the cancer cells and stromal components in pancreatic cancer may represent the treatment strategy required to significantly improve the clinical outcome of this devastating disease.

ACKNOWLEDGEMENTS

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2014 ADVANCES IN PANCREATIC CANCER

Strategies for early detection of resectable pancreatic cancer

Keiichi Okano, Yasuyuki Suzuki

Keiichi Okano, Yasuyuki Suzuki, Department of Gastroenterological Surgery, Faculty of Medicine, Kagawa University, Kagawa 761-0793, Japan
Author contributions: Okano K drafted this manuscript, which was revised by Suzuki K.
Correspondence to: Keiichi Okano, MD, PhD, Department of Gastroenterological Surgery, Faculty of Medicine, Kagawa University, 1750-1, Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. kokano@kms.ac.jp
Telephone: +81-87-8912438 Fax: +81-87-8912439

Core tip: To improve the prognosis of patients with pancreatic cancer, it is essential to detect tumors at early stages, when they are resectable. The cancer of the pancreas screening program has reached several conclusions and recommendations for the management of patients who are at an increased risk of familial pancreatic cancer. Furthermore, genetic, epigenetic, and proteomics research have improved the understanding of the mechanisms of this disease, potentially offering biomarkers that could allow the cancer to be detected early. This article reviews strategies for the early detection of resectable pancreatic cancer.

Abstract

Pancreatic cancer is difficult to diagnose at an early stage and generally has a poor prognosis. Surgical resection is the only potentially curative treatment for pancreatic carcinoma. To improve the prognosis of this disease, it is essential to detect tumors at early stages, when they are resectable. The optimal approach to screening for early pancreatic neoplasia has not been established. The International Cancer of the Pancreas Screening Consortium has recently finalized several recommendations regarding the management of patients who are at an increased risk of familial pancreatic cancer. In addition, there have been notable advances in research on serum markers, tissue markers, gene signatures, and genomic targets of pancreatic cancer. To date, however, no biomarkers have been established in the clinical setting. Advancements in imaging modalities touch all aspects of the clinical management of pancreatic diseases, including the early detection of pancreatic masses, their characterization, and evaluations of tumor resectability. This article reviews strategies for screening high-risk groups, biomarkers, and current advances in imaging modalities for the early detection of resectable pancreatic cancer.

INTRODUCTION

Pancreatic cancer is an especially lethal malignancy, with a mortality rate that almost equals its incidence. After pancreatic cancer is diagnosed, the 1-year relative survival rate is only 24%, and the 5-year overall survival rate is only 5%[1,2]. However, rates of overall survival have been improving over the past decades, for both resected and non-resected cases[1]. These improvements are believed to have resulted from more optimal patient selection, refinements in surgical techniques, and better postoperative patient care, in addition to the development of effective adjuvant therapies. In cases of pancreatic carcinoma,

complete surgical resection with adjuvant chemotherapy offers the best outcomes[3]. However, over 80% of patients with pancreatic cancer present with an unresectable primary tumor and distant metastasis at the time of diagnosis[4]. Of patients with resectable pancreatic cancers, only 15% have earliest-stage cancers (T1 or T2 tumors without lymph node metastases), which are associated with better survival[5,6]. Thus, only 2%-3% of all patients diagnosed with pancreatic cancer present with earliest-stage cancer. Among the patients with pancreatic cancer who undergo surgical resection, the 5-year survival rate is 15%-40%[7]. In a study of operated pancreatic cancers from the Japanese Pancreatic Cancer Registry, it was observed that patients with stage I tumors < 2 cm in size had considerably better survival (58% alive at 5 years) than patients with stage Ⅱb tumors (17% alive at 5 years)[1]. In another study, 100% 5-year survival was observed among 79 patients who had tumors < 1 cm and had undergone curative resection[8].

Recently, a valuable analysis about the timing of the genetic evolution of pancreatic cancer was reported[9]. The authors indicated at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell. Furthermore, at least five more years are required for the acquisition of metastatic ability and patients die an average of two years thereafter. These data provide novel insights into the genetic features underlying pancreatic cancer progression and define a broad time window of opportunity for early detection to prevent deaths from metastatic disease. For these reasons, significant efforts have been invested towards identifying high-risk groups, sensitive biomarkers, and accurate imaging modalities for pancreatic cancer. Each of these advancements can facilitate the early diagnosis of pancreatic cancer that is resectable or potentially resectable.

CURRENT CRITERIA FOR RESECTABILITY

In the absence of metastatic disease, pancreatic cancer cases are classified into three main categories: resectable, borderline resectable, and unresectable. Recent revisions of the National Comprehensive Cancer Network (NCCN) guidelines have attempted to distinguish tumors that are clearly resectable from those that are borderline resectable[10]. Further, the NCCN guidelines provide a definition for radiographically resectable tumors. The specific NCCN guidelines have been quoted below[10].

Tumors considered "resectable" should demonstrate the following (1) No distant metastases; (2) No radiographic evidence of superior mesenteric vein (SMV) or portal vein (PV) distortion; and (3) Clear fat planes around the celiac axis, hepatic artery, and SMA.

Tumors considered "borderline resectable" include the following: (1) No distant metastases; (2) Venous involvement of the SMV or PV with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing safe resection and

replacement; (3) 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; and (4) Tumor abutment of the SMA not to exceed > 180° of the circumference of the vessel wall.

To improve the prognosis of patients with pancreatic cancer, it is essential to detect tumors at early stages, when they are more likely to be resectable.

SCREENING HIGH-RISK GROUPS TO FACILITATE EARLY DIAGNOSIS OF PANCREATIC CANCER

As presented in Table 1, previous studies have identified a variety of risk groups and factors for developing pancreatic cancer. An elevated risk of developing pancreatic cancer is associated with being a current smoker[11], African-American[4], over 55 years old[4], male[4], obese[12], previously diagnosed with intraductal papillary mucinous neoplasms (IPMNs)[13], or previously diagnosed with diabetes[12,14]. Additionally, family history can be used to identify some individuals who have a high risk of developing pancreatic cancer. An increased risk of pancreatic cancer has been linked to family histories of pancreatic cancer[15,16], chronic pancreatitis[17,18], hereditary pancreatitis[19,20], Peutz-Jeghers syndrome[21,22], familial atypical multiple mole melanoma, cystic fibrosis[23], and familial cancer syndromes, which include Lynch syndromes[24,25], familial adenomatous polyposis pAPC mutation, and hereditary breast and ovarian cancer syndrome with *BRCA1* and *BRCA2* mutations[26,27]. This section of our review focuses on screening guidelines, the importance of new-onset diabetes, and the identification of precancerous lesions for the early detection of resectable pancreatic cancer.

Screening programs

The cancer of the pancreas screening (CAPS) program is one of largest pancreatic screening initiatives to date. Results from the CAPS 1 and CAPS 2 studies show that early pancreatic neoplasia can be detected by screening asymptomatic patients[28,29]. In the CAPS 1 study, the diagnostic yield of screening was 5.3%. Most encouragingly, the patient who was diagnosed with pancreatic cancer as a consequence of screening is still alive and disease free more than 5 years after surgery[28]. CAPS 2 screening was performed using annual endoscopic ultrasound (EUS) and computed tomography (CT). Once an abnormality had been detected, endoscopic retrograde cholangiopancreatography (ERCP) was offered. Of the 72 high-risk patients, eight had pancreatic neoplasia confirmed by surgery or fine-needle aspiration biopsy (FNA), constituting a 10% yield of screening. The CAPS 3 study is an ongoing multicenter prospective controlled cohort study that involves annual screening using EUS and magnetic resonance cholangiopancreatography (MRCP).

Table 1 Risk factors for pancreatic cancer

Variables	Association	Ref.
Non-genetic risk factors		
Age	Ages 55-64 yr: 20.7% of cases; ages 65-74 yr: 25.8% of cases; ages 75-84 yr: 27.8% of cases; age 85 + yr: 13.3% of cases	[4]
Gender	The incidence rate is 13.8 per 100000 men and 10.8 per 100000 women	[4]
Smoking	Most established risk factor for PC. Risk increases significantly with greater intensity: $\geq$ 30 cigarettes/day (OR = 1.75, 95%CI: 1.27-22.42); duration $\geq$ 50 yr (OR = 2.13, 95%CI: 1.25-3.62); and cumulative smoking dose $\geq$ 40 pack-years (OR = 1.78, 95%CI: 1.35-2.34)	[11]
Obesity	Obese individuals (BMI $\geq$ 30) have a slightly higher risk (RR: 1.19) of developing PC compared with normal-weight individuals (BMI < 25)	[12]
Race	15.5 males and 12.6 females per 100000 in African-Americans, while 8.4 males and 6.9 females per 100000 for Asians/Pacific Islanders	[4]
Diabetes mellitus (DM)	Meta-analysis from 35 cohort studies revealed a RR ratio of 1.94 (95%CI: 1.66-62.27) between type 2 DM and PC. 40%-100% increases in the risk of PC are observed with established diabetes	[12,14]
New-onset diabetes	New-onset diabetes is associated with a four- to seven-fold increase in risk, such that 1%-2% of patients with recent-onset diabetes will develop PC within 3 yr	[30]
Intraductal papillary mucinous neoplasms	Standardized incidence ratio 16	[13]
Hereditary cancer syndromes		
Familal pancreatic cancer	1 first-degree relative: 4.6-fold increased risk (95%CI: 0.5-16.4); $\geq$ 2 first-degree relatives: 6.4-fold increased risk (95%CI: 1.8-16.4); $\geq$ first-degree relatives: 32-fold increased risk (95%CI: 10.2-74.7)	[15,16]
Chronic pancreatitis	An incidence ratio of 14-18 observed for the development of PC in CP cases, which is further increased by cigarette smoking	[17,18]
Hereditary pancreatitis	A 53-fold (95%CI: 23-105) increased risk for developing PC and a lifetime risk (age 70 yr) of PC of 30%-40% in comparison with normal. RR increases further in smokers	[19,20]
Peuts-Jeghers	132-fold (95%CI: 44-261) increased risk of PC compared with the general population	[21,22]
Lynch syndrome	8.6-fold (95%CI: 4.7-15.7) increased risk for developing PC compared with the general population. An estimated 3.68% (95%CI: 1.45-45.88%) lifetime (age 70 yr) risk of PC	[24,25]
Hereditary breast and ovarian cancer	BRCA2 germline mutation carriers have a 5% lifetime risk of PC in comparison with 1.78% for controls. BRCA1 mutation is 2.26-times that of the normal population	[26,27]

PC: Pancreatic cancer.

CAPS 3 is also investigating magnetic resonance imaging (MRI) with secretin and a panel of candidate DNA and protein markers (in serum and pancreatic juice) as indicators of pancreatic neoplasms. Carbohydrate antigen 19-9 (CA19-9), macrophage inhibitory cytokine-1 (MIC-1), DNA hypermethylation, and *K-ras* gene mutations are presently under investigation as potential markers. The CAPS consortium has reached several conclusions and recommendations for the management of patients who are at an increased risk of familial pancreatic cancer[16]. The CAPS consortium specifically agreed that the following individuals were candidates for screening: first-degree relatives (FDRs) of patients with pancreatic cancer in a familial pancreatic cancer kindred with at least two affected FDRs; patients with Peutz-Jeghers syndrome; and carriers of *p16, BRCA2*, and hereditary non-polyposis colorectal cancer (HNPCC) mutations with at least one affected FDR. The consortium agreed that initial screening should include EUS, potentially with MRI or MRCP, but excluding CT and ERCP. The consortium did not agree on optimal screening modalities, intervals for follow-up imaging, or the use of EUS-FNA to evaluate cysts.

In general, screening was recommended for high-risk individuals. However, additional evidence is needed regarding the sensitivity and cost-effectiveness of screening, as well as the choice of management strategy for patients with lesions that are detected by screening.

New-onset diabetes

The CAPS approach does not contribute to the early detection of pancreatic cancers that have completely sporadic onsets. To identify early pancreatic cancers in sporadic groups, it may be possible to screen patients at the onset of diabetes mellitus. The new onset of diabetes mellitus is occasionally associated with pancreatic carcinoma that is otherwise clinically silent and, indeed, is potentially resectable[30]. A population-based cohort study of 2122 diabetic individuals identified 18 (0.8%) patients who developed diabetes at age 50 years or older and were diagnosed with pancreatic cancer in the next 3 years. In this cohort of individuals who were newly diagnosed with diabetes, the ratio of observed-to-expected pancreatic cancer incidence was 7.9 (95%CI: 4.7-12.5)[31].

Diabetes is highly prevalent in cases of pancreatic cancer, even for early-stage pancreatic cancers[32-36]. Specifically, 50% of patients with stage I or II pancreatic cancer had diabetes[37]. Tsuchiya *et al*[36] observed abnormal glucose tolerance in 61% of patients with small pancreatic cancers ($\leq$ 2 cm). A study of especially small pancreatic cancers (< 10 mm) noted a 33% prevalence of diabetes[38]. Because diabetes arises in almost half of patients with pancreatic cancer, it is an attractive target for early pancreatic cancer screening.

Identification of precancerous lesions

Precancerous lesions are ideal targets for early identification because they can be treated before developing into invasive cancer. The majority of pancreatic masses treated by surgical resection are IPMNs, which have been increasingly recognized as precursors to pancreatic ductal adenocarcinoma[39]. Post-resection cure rates are very high for IPMN that does not have an associated infiltrating ductal pancreatic adenocarcinoma[40,41]. Pancreatic intraepithelial neoplasias (PanINs) are small neoplasms ($\leq$ 5 mm) that are mostly found in the head of the gland and are thought to be the most common precursor to invasive pancreatic ductal adenocarcinoma[39]. Most precancerous lesions (and especially PanINs) can only be identified reliably after surgical resection. Because many healthy individuals have low-grade PanINs that will never progress to clinically important neoplasms[42], markers are needed to help differentiate between neoplastic and non-neoplastic pancreatic lesions, as well as to indicate the presence of microscopic high-grade PanINs that might be suggestive of future pancreatic cancer risk.

The most challenging aspect of screening and surveillance programs is the management of asymptomatic pancreatic lesions that are detected by imaging tests. It is essential to have individualized decision-making within multidisciplinary programs and prospective research studies.

BIOMARKERS THAT FACILITATE EARLY DIAGNOSIS OF PANCREATIC CANCER

Biomarker screening is one possible approach for identifying these early lesions. To date, over 2000 studies of possible biomarkers have been published[43]. Yet, biomarkers for the detection of small pancreatic cancer have not been validated.

Serum markers

CA19-9 is a sialylated Lewis (a) antigen; it is a carbohydrate that is produced by exocrine epithelial cells and is normally absorbed onto erythrocyte surfaces. The measurement of CA19-9 levels has never been shown to be effective as a screening test for pancreatic cancer. In a study of 10162 asymptomatic individuals, abnormal CA19-9 levels were identified in only 18 (0.2%) persons[44]. Although this study used a variety of screening tests, only four pancreatic cancers (0.04%) were detected. Pleskow *et al*[45] performed one of the first studies that established CA19-9 as a promising biomarker in pancreatic cancer. In this study of 261 patients (including 54 with pancreatic cancer), the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of CA19-9 were 70%, 87%, 59%, 92% and 84%, respectively. In addition, preoperative CA19-9 test levels constitute false positives in the setting of biliary obstruction, which is present in the majority of patients with pancreatic cancer and various benign conditions related to the pancreas and biliary tract[46]. There is some evidence that preoperative CA19-9 measurements can help determine whether a pancreatic cancer is resectable[47]. Maithel *et al*[48] reported a strong association between preoperative CA19-9 values and the identification of unresectable pancreatic cancer that could not be recognized on diagnostic imaging studies. They recommend staging laparoscopy for pancreatic cancers associated with CA19-9 levels that exceed 130 U/mL.

Carbohydrate antigens of mucin-1 (MUC-1) have been investigated as potential means of improving on the performance of CA19-9[49]. Yet, none of the assays used to detect MUC-1 carbohydrate epitopes have proven to be superior to CA19-9 measurements. PAM-4 can be used to detect MUC-1 proteins expressed in pancreatic cancer with a greater specificity than MUC-1 proteins expressed in other cancers[50]. Additionally, initial studies have shown that an enzyme-linked immunosorbent assay directed at detecting circulating MUC-1 epitopes is more sensitive and specific than CA19-9 for identifying patients with pancreatic cancer[50].

In a recent study, serum MIC-1 was determined to be more sensitive than CA19-9 as a marker of pancreatic cancer[51]. MIC-1 belongs to the transforming growth factor-β superfamily, which was first identified in the context of macrophage activation[52]. MIC-1 is overexpressed in pancreatic, colon, prostrate, breast, gastric, and several other types of cancers[53-55], and therefore, it may prove useful for diagnosing other cancers[56]. In an investigation of pancreatic cancer and MIC-1 levels, 90% of patients with resectable pancreatic cancer had MIC-1 levels that were more than 2 standard deviations greater than those in age-matched controls. By comparison, only 62% of patients with resectable pancreatic cancer had elevated CA19-9. Elevated MIC-1 was observed to be independent of TNM stage. Further, elevated MIC-1 was observed in six of seven patients who had T1 or T2 cancers, but elevated CA19-9 was observed in only two of these seven patients[57]. Based on these findings, serum MIC-1 may prove to be useful as a component of pancreatic screening protocols for detecting early stage pancreatic cancers in high-risk groups[28,58].

Proteomics

Proteomics approaches have also been employed in an attempt to identify protein markers of pancreatic cancer[59-62]. Several groups have identified protein fragments in serum using surface-enhanced laser desorption ionization, which appears to have found protein fragments that function as diagnostic makers at least as effectively as does serum CA19-9[63,64]. Pancreatic cancer proteins have also been identified in serum using matrix-associated laser desorption ionization, which is another mass spectrometry approach[65]. Proteomics studies have identified several important proteins that are associated with pancreatic tumorigenesis, including galectin-1, gelsolin, lumican, 14-3-3 protein sigma, cathepsin D, cofilin, moesin, and plectin-1[60,66,67]. Gelsolin and lumican were later tested in plasma, showing an 80% sensitivity and a 95% specificity

as a composite biomarker for separating early stage pancreatic cancer patients (stages Ⅰ and Ⅱ) from healthy controls and patients with chronic pancreatitis (*via* selected reaction-monitoring-based targeted proteomics assays)[68]. The application of proteomics to the study of pancreatic cancer is still in its early stages and remains challenging. Yet, despite being an emerging technology, proteomics has already provided fundamental information that has improved our understanding of this disease's mechanisms. Further, proteomics potentially offers solutions for the early detection of this cancer.

Genetic and epigenetic markers

K-ras mutations are present in up to 90% of pancreatic ductal adenocarcinomas[69,70]. Accordingly, *K-ras* mutants have been thoroughly investigated as markers of pancreatic adenocarcinoma. In addition to invasive pancreatic cancers, K-ras mutations also occur in patients with chronic pancreatitis, persons who smoke, and PanINs in patients who do not have pancreatic cancer[69]. Additionally, mutant K-ras is detected in the blood of patients with advanced-stage pancreatic cancers more commonly than it is detected in the blood of patients with less advanced pancreatic cancers[71,72].

TP53 mutations have been extensively investigated as possible diagnostic markers of a variety of cancers. In the case of invasive pancreatic cancer, however, such mutations do not normally occur until late in the neoplastic process. *TP53* gene mutations are found in 70% of invasive pancreatic ductal adenocarcinomas[73]. Mutations occur throughout the *TP53* gene, although several nucleotide hot spots have been identified, at which mutations are especially common[74].

The strategy of combining markers can optimize the diagnosis of pancreatic cancer through molecular examination[75]. In a study of a combined marker panel, the combination of methylated p16, mutant K-ras, and a functional yeast assay for *TP53* mutations was investigated[75]. The authors concluded that the presence of *TP53* mutations was the most specific. With improvements in the technology for detecting mutations, *TP53* mutations in pancreatic juice may underpin an effective diagnostic strategy.

Pancreatic cancer is both a genetic and an epigenetic disease[76,77]. Various genes are methylated as pancreatic cancer arises, and non-neoplastic pancreatic tissues rarely show methylation of these same genes. Genes that are methylated in the process of pancreatic cancer formation are p16[78], RELN[79], DAB1[79], ppENK[80], Cyclin D2[81], SOCS1[82], *SPARC*[83], TSLC1[84], and others[85,86]. Because the methylation of some of these genes can be detected through methylation-specific polymerase chain reaction, and because some of these genes are also highly expressed in pancreatic cancers, epigenetic markers may provide an opportunity for the early detection of pancreatic cancers.

Other potential markers

Promising biomarkers have also been established for pre-

dicting the effectiveness of chemotherapy and immune-based therapy. The human equilibrative nucleoside transporter (hENT1) protein transports gemcitabine into cells. In a prospective randomized trial (RTOG9704), hENT1 protein expression was associated with increased overall survival and disease-free survival in pancreatic cancer patients who received gemcitabine, but not in those who received fluorouracil. These findings are supported by preclinical data; the gemcitabine transporter hENT1 is therefore a molecular and mechanistically relevant predictive marker of benefit from gemcitabine in patients with resected pancreatic cancer[87]. In addition to hENT1, key determinants of gemcitabine cytotoxicity include the activities of deoxycytidine kinase (dCK). Indeed, high levels of hENT1 and dCK predict longer survival times in patients with pancreatic cancer who are treated with adjuvant gemcitabine[88].

Mesothelin is a glycoprotein expressed on normal mesothelial cells. It is overexpressed in several histologic types of tumors, including pancreatic adenocarcinomas. A soluble form of mesothelin has been detected in patients with ovarian cancer and malignant mesothelioma, and has been found to have prognostic value. Circulating mesothelin is also a useful biomarker for pancreatic cancer. Furthermore, mesothelin-specific T cells can be induced in patients with pancreatic cancer. This suggests that mesothelin is a potential target for immune-based intervention strategies in pancreatic cancer[89]. Although it is not yet clear how these markers specifically relate to the early diagnosis of pancreatic cancer, they may be clinically useful for treatment selection.

Investigations of pancreatic juice have involved both genetic and epigenetic markers for pancreatic cancer. To date, mutant K-ras, p53 mutations, DNA methylation alterations, mitochondrial DNA mutations, and other potential genetic and epigenetic markers have been investigated in pancreatic juice[75]. The MitoChip allows investigations of the mitochondrial genome. Early studies using this novel technology suggest that it can be used to detect mitochondrial mutations in pancreatic juice samples that are taken from patients with pancreatic cancer[90].

Genetic, epigenetic, and proteomics research have improved the understanding of the mechanisms of pancreatic cancer, potentially offering biomarkers that could allow its early detection. It is critically important to validate the utility of these biomarkers in clinical setting as soon as possible.

IMAGING FOR THE EARLY DIAGNOSIS OF PANCREATIC CANCER

Every aspect of the clinical management of pancreatic diseases is influenced by imaging studies. Specific examples include the early detection and characterization of pancreatic masses, the identification of anatomical variants, investigations of local and vascular involvement, the determination of perineural and lymphatic invasion, margin assessments, the detection of distant metastases,

and assessments of tumor resectabilty[91]. Because effective screening markers remain elusive, imaging remains the primary form of screening for cases of familial pancreatic cancer, in addition to its more routine use in the staging and management of pancreatic cancer[28,29,92-94]. Recently, imaging accuracy has been improving as a result of technological improvements. However, imaging still fails to detect many lesions that are under a centimeter in size.

EUS

In comparison with other approaches to imaging, EUS has been growing in popularity. Indeed, EUS offers a large variety of benefits. First, it can detect pancreatic lesions and intraductal papillary mucinous neoplasms that are less than a centimeter in size with a greater sensitivity than is offered by abdominal ultrasonography, CT, or MRI. Second, EUS accurately judges deep tumors. Third, EUS-guided FNA enables lesion biopsies and has an excellent diagnostic accuracy (92%)[95]. Fourth, EUS detects lymph node metastasis and vascular infiltration with greater sensitivities than are offered by CT imaging. More specifically, advancements in contrast-enhanced EUS technology could improve the characterization of vessels in the desired lesions, the accuracy of tumor staging, the accuracy of tumor follow-up, and differential diagnosis. Additionally, improvements in EUS elastography could advance real-time evaluations of tissue stiffness. Finally, hybrid imaging (such as CT/ultrasonography or CT/ultrasonography/MRI) may offer an opportunity to improve the detection and characterization of focal lesions[96].

For lesions < 2 cm, EUS is associated with a sensitivity and accuracy that approach 100%, as well as a specificity > 95%[97-100]. In an analysis of EUS-FNA for pancreatic lesions < 3 cm, Tadic *et al*[101] demonstrated a sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of 68%, 100%, 100%, 73%, and 83%, respectively. Based on these results, it appears that EUS has become quite capable of providing histological evidence, for which there is a great needed. Therefore, EUS should be performed wherever sufficient expertise is available.

Multi-detector CT

The resolution and diagnostic capabilities of CT scanners have improved to remarkable extents. Currently, 64-section thin-cut intravenous contrast-enhanced multi-detector CT (MDCT) is the tool of choice for radiological investigations. Scanning occurs in a sequence of phases: non-contrast, arterial, pancreatic parenchymal, and portal venous. Key features of MDCT are its rapid anatomic coverage and excellent spatial resolution[102]. When employed for the detection of pancreatic cancers, the sensitivity of CT ranges from 75% to 100%, and its specificity ranges from 70% to 100%[97,99,102-105]. Yet, for lesions ⩽ 2 cm in size, this sensitivity diminishes to 68%-77%[97,103], with an accuracy of 77%[99].

The diagnosis of small pancreatic carcinoma is aided by findings of dilatation of the main pancreatic duct (MPD) and associated pancreatitis[106]. In the case of associated pancreatitis, a contrasting effect is evident between the areas of the pancreatic parenchyma proximal and distal to the site of the MPD obstruction[107,108].

MRI/MRCP

CT and MRI/MRCP are the primary investigations that are most commonly performed for the diagnosis and staging of pancreatic cancers. The choice between CT and MRI/MRCP is generally determined by the availability these individual modalities at medical centers, as well as the availability of the technical expertise that is necessary for interpreting and reporting their results. Fusari *et al*[109] found that, for the diagnosis of pancreatic cancer, MRCP offered a sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of 100%, 88%, 98%, 97%, and 100%, respectively. They also found that MRCP, when evaluating the resectability of pancreatic carcinomas, offered a sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of 88%, 100%, 90%, 100%, and 70%, respectively. As outlined by Miller *et al*[100], the addition of MRCP to CT can offer substantial benefits to tumor diagnosis and staging in several contexts. MRI's excellent contrast resolution is beneficial for detecting small tumors on gadolinium-enhanced fat-suppressed images.

PET

PET is a functional imaging modality that can detect metabolic alterations in tumors, which may precede notable morphological alterations. The radioactive tracer ^{18}F-fluorodeoxyglucose (FDG) has been used extensively in the PET imaging of malignant tumors. PET/CT can accurately detect small primary pancreatic lesions, distant metastases, and post-surgery recurrences. As a result of these capabilities, PET/CT has become increasingly important in the diagnosis and management of pancreatic cancer[110-112]. Elevated glucose metabolism has been found in the precursor lesions of pancreatic cancer, which suggests that there may be an opportunity to detect these changes using PET/CT, and thereby improve the timeliness of diagnosis and patient outcomes[113].

We have previously investigated the role of FDG-PET with dual-time point evaluation in cases of small pancreatic cancer[114]. When investigated using FDG-PET with dual-time point evaluation, all TS1 tumors (< 20 mm) had higher standardized uptake values in the delayed phase than in the early phase, which suggested that the lesions were malignant tumors. These results indicate that FDG-PET with dual-time point evaluation is a useful modality for diagnosing small pancreatic cancers.

A recent meta-analysis[115] regarding the detection of pancreatic carcinoma found a pooled sensitivity of 90.1% for PET-CT, which was substantially better than the 81.2% pooled sensitivity of EUS. However, PET-CT was also associated with a pooled specificity of 80.1%,

while EUS had a pooled specificity of 92.3%. These results are similar to the findings of two previously published reviews of the literature on the same topic[116,117]. The role of FDG-PET in the early detection and accurate staging of pancreatic cancer is controversial. We suggest that future research should definitely focus on the development of more specific PET tracers for pancreatic ductal adenocarcinoma.

CONCLUSION

Despite advancements in surgical techniques and adjuvant treatment, the prognosis of pancreatic cancer has only improved marginally over the past years. Future research should continue and expand recent investigations of screening for high-risk groups, sensitive biomarkers, and imaging modalities for the early diagnosis of resectable pancreatic cancer. Recent studies have successfully identified pre-invasive neoplasms using accurate pancreatic imaging tests. These advancements are encouraging. They attest to the importance of additional studies that are aimed at identifying individuals at a substantially increased risk of developing pancreatic neoplasia.

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2014 ADVANCES IN PANCREATIC CANCER

Advances in pancreatic cancer research: Moving towards early detection

Xiang-Yi He, Yao-Zong Yuan

Xiang-Yi He, Yao-Zong Yuan, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China
Author contributions: He XY collected all the references and drafted the article; Yuan YZ chose the topic, organized the structure and revised the article.
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Correspondence to: Yao-Zong Yuan, MD, PhD, Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Er Road, Shanghai 200025, China. yyz28@medmail.com.cn
Telephone: +86-21-64150773 Fax: +86-21-64150773
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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer. Substantial progress has been made in the understanding of the biology of pancreatic cancer, and advances in patient management have been significant. However, most patients (nearly 80%) who present with locally advanced or metastatic disease have an extremely poor prognosis. Survival is better for those with malignant disease localized to the pancreas, because surgical resection at present offers the only chance of cure. Therefore, the early detection of pancreatic cancer may benefit patients with PDAC. However, its low rate of incidence and the limitations of current screening strategies make early detection difficult. Recent advances in the understanding of the pathogenesis of PDAC suggest that it is possible to detect PDAC in early stages and even identify precursor lesions. The presence of new-onset diabetes mellitus in the early phase of pancreatic cancer may provide clues for its early diagnosis. Advances in the identification of novel circulating biomarkers including serological signatures, autoantibodies, epigenetic markers, circulating tumor cells and microRNAs suggest that they can be used as potential tools for the screening of precursors and early stage PDAC in the future. However, proper screening strategies based on effective screening methodologies need to be tested for clinical application.

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Key words: Pancreatic ductal adenocarcinoma; Early detection; Diabetes mellitus; Pancreatic cancer

Core tip: Because pancreatic cancer is usually detected at an advanced stage and there is a lack of treatment strategies for advanced disease, it remains one of the most lethal solid tumors. Genetic and epigenetic alterations, miRNAs and tumor microenvironment promote the development of pancreatic cancer from precursor lesions to localized disease and further to metastatic disease in several years. An effective screening strategy for pancreatic cancer is therefore needed. New-onset diabetes mellitus associated with pancreatic cancer and recently identified novel circulating biomarkers should be explored as potential screening markers.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer. It is the 13th most commonly diagnosed cancer worldwide[1], and the eighth lead-

ing cause of cancer death. No early detection tests are available and most patients with localized disease have no recognizable symptoms or signs; as a result, most patients (80%-85%) are not diagnosed until late in the disease, when the cancer has metastasized to other organs[2,3]. Survival is better for those with malignant disease localized to the pancreas, because at present, surgical resection offers the only chance of cure. Because pancreatic cancer responds poorly to radiation and chemotherapy, and so far most of the targeted therapy agents have failed to show a substantial benefit, PDAC in the advanced stage is associated with an extremely poor prognosis[2,3]. Therefore, the early detection of PDAC has gained increasing attention with the aim of improving the outcomes of patients with this disease. Recent studies have improved our understanding of the pathogenesis of PDAC, the relationship between diabetes mellitus (DM) and PDAC, and the role of circulating biomarkers. The present review will discuss the possibility of using these data to detect PDAC in its early stage and propose future research directions.

RECENT ADVANCES IN THE UNDERSTANDING OF THE PATHOGENESIS OF PDAC

Despite the short lifespan of patients diagnosed with PDAC, the disease usually develops over a long period of time. Based on a genetic evolutionary model[4], it is estimated that 10-30 years are required from the initiating mutation until the patient's death. In this model, there are three critical periods in the genetic evolution of the disease: T1 is related to the formation of precursor lesions such as pancreatic intraepithelial neoplasia (PanIN) and lasts until the infiltrating carcinoma is first formed; T2 describes the period from that time until a metastatic subclone develops within the primary carcinoma; and T3 is the period of metastatic dissemination of that subclone until the patient's death. A conservative estimate of 11.7, 6.8 and 2.7 years per interval, respectively, has been reported[4].

The development of PDAC involves multiple steps. It may originate from four distinct precursors, *i.e.*, mucinous cystic neoplasm (MCN), intraductal papillary mucinous neoplasm (IPMN), PanIN, and the newly added intraductal tubular papillary neoplasms (ITPNs)[5]. All four harbor varying degrees of dysplasia and stepwise accumulation of genetic alterations, suggesting progression of these lesions from benign toward malignant neoplasms. MCNs have a characteristic ovarian-type stroma. Approximately one-third of MCNs are associated with invasive carcinoma of a ductal phenotype. IPMNs are recently established clinical entities with characteristic features of mucin hypersecretion and duct dilatation. Some IPMNs are associated with invasive carcinoma and IPMNs are recognized as precursors to pancreatic cancer. ITPNs are rare premalignant tumours, with a concomitant invasive

component, and were included for the first time in the 2010 World Health Organization classification[5]. PanINs are microscopic proliferative lesions arising from any part of the pancreatic duct system. Low grade PanINs are commonly found in the pancreatic ducts of older individuals, whereas high grade PanINs, previously called carcinoma *in situ*/severe ductal dysplasia, may eventually give rise to invasive pancreatic cancer. PanINs are divided into four categories based on the degree of dysplasia (1A, 1B, 2, and 3). Appropriate clinical management is critical for patients with MCNs, IPMNs and PanINs. The progression from PanIN to invasive PDAC has been intensively studied and reviewed elsewhere[6]. The established model describes the stepwise progression of PanINs to PDAC through the accumulation of several important genetic aberrations. KRAS mutation may be the first step driving this progression, and it is detected in approximately 99% of PanIN-1 lesions[7]. In addition, to overcome oncogene-induced senescence, loss of function of CDKN2A, and genetic inactivation of TP53, SMAD4, and BRCA2 are also required for the development of PDAC[8]. Further investigation of these precursor lesions is expected to reduce the mortality from pancreatic cancer.

The development of PDAC involves multiple genes. In 2008, detailed, global, genomic analyses found that a large number of genetic alterations (an average of 63) affect a core set of 12 signaling pathways and processes that are each genetically altered in 67%-100% of cases of pancreatic cancer[9]. However, the pathway components that are altered in any individual tumor vary widely. The 12 core pathways[9] are apoptosis, DNA damage control, regulation of G1/S phase transition, Hedgehog signaling, homophilic cell adhesion, integrin signaling, c-Jun N-terminal kinase signaling, KRAS signaling, regulation of invasion, small GTPase-dependent signaling (other than KRAS), TGF-β signaling and Wnt/Notch signaling. In addition to the core pathways described above, more recent data indicate that chromatin regulation[10] and axon guidance[10,11] are additional cellular processes that play a crucial role in pancreatic cancer. The pathways involved in each patient often vary. This finding may help account for the heterogeneous nature of tumors and offer insights into why agents targeting a specific gene in a pathway rarely result in a therapeutic advantage in more than a minor percentage of patients. Therefore, identification of the core players of each pathway and the factors connecting them is important. In addition to whole genomic alterations, numerous investigators have focused on particular PDAC-associated genes through laboratory and patient studies. Our group demonstrated that Periostin[12], X chromosome-linked inhibitor of apoptosis (XIAP)-associated, factor 1 (XAF1, a novel XIAP modulator)[13], angiotensin-converting enzyme 2[14], bone morphogenetic protein-2[15], eEF1A2[16], L1 cell adhesion molecule (L1-CAM)[17], and DJ-1[18] are associated with PDAC proliferation, apoptosis, invasion and/or progression.

MicroRNAs (miRNAs) are a highly conserved family of 18-24-nucleotide RNA molecules that regulate the sta-

bility or translational efficiency of complementary target mRNAs. More than 20 miRNAs involved in pancreatic adenocarcinoma biology have been identified and shown to affect tumor growth, metastatic potential, and chemosensitivity[19]. Combinations of miRNAs can be used to differentiate between pancreatic adenocarcinoma and other pancreatic pathologies, as well as to assess prognosis. Manipulations of miRNAs can decrease the rate of growth or reinstall chemosensitivity to certain chemotherapeutic agents. The most extensively studied miRNA, as far as pancreatic cancer is concerned, is miR-21, which has been associated with cell proliferation, metastatic ability, decreased gemcitabine sensitivity, and poor overall survival (OS)[20-23]. Other PDAC associated miRNAs were discussed elsewhere. Recently, several other miRNAs (miR-196a[24,25], 130b[26], 92a[27], 198[28], 221[29,30], 23b[31], 29a[32,33]) were shown to play an important role in PDAC.

Tumors are complex tissues in which mutant cancer cells have conscripted and subverted normal cell types to serve as active collaborators in their neoplastic agenda. Recent studies have shown that PDAC is one of the most stroma-rich cancers. The tumor microenvironment surrounds most of the tumor mass and consists of a dynamic assortment of extracellular matrix components and non-neoplastic cells including fibroblastic, vascular and immune cells. Recent work has revealed that the PDAC stroma supports tumor growth and promotes metastasis, and simultaneously serves as a physical barrier to drug delivery[34]. Pancreatic stellate cells (PSCs) identified in 1998, have the ability to trans-differentiate from a "quiescent" retinoid/lipid storing phenotype in the normal pancreas to an "activated" a-smooth muscle actin producing myofibroblastic phenotype[35]. The activated PSCs produce the extracellular matrix proteins that comprise the pancreatic tumor stroma, to facilitate pancreatic cancer development[35]. Sonic hedgehog signaling has been shown to be restricted to the stromal compartment and enhance the desmoplastic reaction[36]. Findings[36] suggest that increased HIF-1α produced by hypoxic tumors triggers the desmoplastic reaction in pancreatic cancer.

The genetic evolutionary model of PDAC suggests a detection window of several years (T1 + T2) for this disease. The visualization of PDAC precursor lesions using currently available imaging methods is limited; therefore, the detection of precursors is difficult. Improving our understanding of the mechanisms of precursors and PDAC development may help identify tumor biomarkers for this disease.

DM AND PDAC

Because of the low incidence of PDAC in the general population, population-based screening is not recommended. It is more practical to screen individuals at increased risk for PC based on their family history or identifiable genetic predisposition, or patients with diseases known to increase the risk of pancreatic cancer, such as chronic pancreatitis and type Ⅱ DM. Patients with a family history of pancreatic cancer or mutation carriers (germline mutations in the *BRCA2*, *PALB2*, *p16*, *STK11*, *ATM*, *PRSS1* genes and Lynch syndrome or Peutz-Jeghers syndrome) should be screened for pancreatic cancer according to the recommendations of the International Cancer of the Pancreas Screening consortium[37]. Here, we will discuss the relationship between DM and PDAC and the possibility of using new-onset DM as a marker for the detection of PDAC.

Increasing evidence suggests that DM is related to PDAC. It is now recognized that although long-standing diabetes is an etiological factor for pancreatic cancer, new-onset diabetes is its manifestation[38-40]. Epidemiological investigations have found that long-term type 2 DM is associated with a 1.5-fold to 2.0-fold increase in the risk of pancreatic cancer[40]. The evidence suggesting that new-onset diabetes is the manifestation of PDAC, or in other words, caused by PDAC, is that: (1) new-onset diabetes is associated with a high prevalence of PDAC; (2) diabetes associated with pancreatic cancer is predominantly new-onset; (3) pancreatic cancer resection ameliorates diabetes; and (4) experimental data. A meta-analysis[41] conducted in 2005 that included 17 case-control and 19 cohort and nested case-control studies published between 1996 and 2005 demonstrated that the combined age-adjusted and sex-adjusted odds ratio (OR) for pancreatic cancer associated with diabetes was 1.8 (95%CI: 1.7-1.9) and was lower still (OR = 1.5) in patients with a ≥ 5 year history of diabetes. In a pooled analysis[42] of 2192 patients with pancreatic cancer and 5113 cancer-free controls in three large case-control studies conducted in the United States, diabetes was associated with a 1.8-fold increase in the risk of pancreatic cancer (95%CI: 1.5-2.1). Risk estimates decreased as the number of years with diabetes increased[42]. A study from our group[43] included 1458 patients with PDAC and 1528 age-, sex- and sociodemographic matched controls and showed that compared with controls, patients with long-standing diabetes (≥ 2-year duration) had a moderately increased risk of PDAC, with an OR (95%CI) of 2.11 (1.51-2.94). Interestingly, a significantly higher risk was observed among cases with new-onset DM (< 2-year duration), with an OR of 4.43 (3.44-5.72) compared to controls without DM. On the other hand, the reported prevalence of DM in pancreatic cancer varies from 23% to 75%, with the majority being new-onset[44]. Our data also showed that 44.7% of PDAC patients harbor DM, and almost 2/3 are new-onset[45]. Data from our group and other groups showed that resolution of DM after pancreatic resection occurs in 41%-57% of PDAC patients with new-onset DM, in contrast to most patients with longstanding DM, who remain diabetic postoperatively[44-46]. Recognition of new-onset diabetes as an early manifestation of pancreatic cancer could improve the detection of asymptomatic, early-stage pancreatic cancer[38].

The impact of DM on the long-term outcomes of patients with PDAC has also been intensively studied recently, although the results are controversial. In patients

with stage I -IV pancreatic cancers, DM does not confer a worse prognosis; in fact, diabetics have a statistically significantly superior median survival[47]. A retrospective study of 344 patients who underwent surgical resection of pancreatic cancers showed that perineural invasion was significantly more common in diabetics, with a poor OS[47]. A multi-institutional retrospective study[48] reported a shorter OS and disease-free survival (DFS) in patients with preoperative DM. By stratifying DM into different groups (long-term/new-onset pre-surgical diabetes, resolved/unresolved post-surgical diabetes), we found that the heterogynous DM groups have different impacts on PDAC outcomes: longstanding DM is predictive of poor postoperative DFS and OS, whereas postoperatively resolved new-onset DM is associated with longer DFS and OS[45].

There are several possible mechanisms to explain the effect of diabetes on promoting PDAC progression, including the cellular proliferative effects of hyperglycemia, hyperinsulinemia, abnormalities in insulin/IGF receptor pathways, oxidative stress and inflammatory responses[40]. A prospective nested case-control study that included 449 case patients and 982 control subjects showed that the highest and the lowest quintiles of HbA1c, insulin, and proinsulin were associated with an increased risk for pancreatic cancer. However, in mutually adjusted models, only circulating markers of peripheral insulin resistance (proinsulin), rather than hyperglycemia (HbA1c) or pancreatic β-cell dysfunction (insulin), were independently associated with pancreatic cancer risk[49]. By comparing the proteome of PDAC with and without DM, our previous study indicated that regenerating gene (REG) I α may be one of the connections between DM and PDAC[50]. The number of REG I α positive cancer cells was significantly higher in pancreatic cancer patients with diabetes (*n* = 38) than in subjects without diabetes. Overexpression of the REG I α protein in pancreatic cancer cell lines resulted in accelerated cell proliferation and consequently tumor growth, both *in vitro* and *in vivo*[50]. The IQ motif containing GTPase activating protein 1-exocyst axis is a growth factor- and nutrient-sensor that couples cell growth and division. It may function at the interface of cancer and diabetes[51].

Several preclinical and observational studies have shown that anti-diabetic medications may modify the risk of pancreatic cancer. A case-control study showed that diabetics treated with metformin had a significantly lower risk of pancreatic cancer (OR = 0.38; 95%CI: 0.22-0.69, *P* = 0.001)[52]. Metformin significantly decreased pancreatic cell growth[53]. These effects could be attributed to disruption of the crosstalk between insulin receptor and GPCR signaling[54], or up-regulation of miR-26a or other factors[53]. However, in a recent meta-analysis that included eleven studies, 1770 cases of pancreatic cancer in 730664 patients with DM were reported, indicating no significant association between metformin, insulin, or TZD use and risk of developing pancreatic cancer, and use of sulfonylureas was associated with a 70% increase in the risk of developing pancreatic cancer[55].

CIRCULATING BIOMARKERS FOR PDAC

Because PDAC develops over a long period of time and the curative response is significantly better in patients with early disease, an early diagnostic marker could positively impact the outcome of patients. Circulating biomarkers are always preferred over others because of their ease of collection and relatively noninvasive nature. The current standard serum marker, sialylated Lewis blood group antigen CA19-9, is widely used, but its use is limited to monitoring responses to therapy and not as a diagnostic marker because of its poor sensitivity (41%-86%) and specificity (33%-100%)[56]. CA19-9 can arise among patients with benign pancreaticobiliary disorders, notably cholestasis, and 5% to 10% of the population does not express Lewis antigens.

In the last two decades, many biomarkers have been tested for PDAC detection, some of which have higher specificity and sensitivity than CA19-9. These are new antibodies such as PAM4 recognizing MUC-1[57], soluble iC3b[58], REG4[59], serum phosphoproteins extracellular signal-regulated kinase (p-ERK1/2)[60], CEACAM1, a proliferation-inducing ligand[61], DJ-1[62], and laminin, gamma 2[63]. Further validation studies including a large number of cases are required for the clinical application of these biomarkers.

DJ-1 is up-regulated in 68.5% of PDAC specimens and correlates with tumor invasion and metastasis. The secretion of DJ-1 by tumor cells implies its potential as a biomarker[64]. Our data showed that the area under the curve (AUC) of serum DJ-1 is higher than that of CA 19-9 in certain patients with PDAC, and serum DJ-1 level also predicts poor patient outcome. Other groups confirmed our results[65] and showed the increase of DJ-1 in pancreatic juice[66].

The sensitivity and specificity of these biomarkers, which may be insufficient when used alone, can be improved by using them in combination or together with CA19-9. In a recent study[67], CA19.9 showed a better AUC in combination with SYCN, REG1B and AGR2 than when used alone. When analyzed in combination, three panels [CA19.9 + REG1B (AUC of 0.88), CA19.9 + SYCN + REG1B (AUC of 0.87) and CA19.9 + AGR2 + REG1B (AUC of 0.87)] showed a significantly better AUC (*P* < 0.05) than that of CA19.9 alone (AUC of 0.82). The superiority of the combination of biomarkers was also shown by our group and others[64,68].

Several recent reports showed that aberrant miRNA production is an early event in the development of PanIN lesions[69,70]. MiRNAs 21[71,72], 155[71], 16[73], 196a[73], 1290[74], 221[30], 375 (lower in PDAC)[30], and 18a[75] were identified by using miRNA expression profiling or other methods as potential biomarkers of PDAC alone or in combination with CA19-9 and each other[72,73]. A recent meta analysis of three blood based miRNA studies reported that the median specificity and sensitivity were 0.91 and 0.96, respectively[72]. Our group together with other centers[71] in China screened differentially expressed serum miRNAs with Illumina's sequencing by synthesis technology

using pooled serum samples followed by RT-qPCR validation of a large number of samples arranged in multiple stages in 97 PDAC cases and 158 age- and sex-matched cancer-free controls. We established 7 miRNA-based biomarker model (miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185, and miR-191) for PDAC diagnosis. These biomarkers had high sensitivity and specificity for distinguishing various stages of pancreatic cancer from cancer-free controls and also accurately discriminated pancreatic cancer patients from chronic pancreatitis patients (AUC = 0.993). In 26 stage I pancreatic cancer cases, the positive rate of pancreatic cancer detection by the 7 miRNA-based biomarker set was 96.2%, which was significantly higher than that of CA19-9 (46.2%) or CEA (30.8%) in the same sample set. In 48 stage II pancreatic cancer cases, the positive rate was 91.7%, which was also higher than that of CA19-9 (62.5%) or CEA (31.3%). Although miRNA detection is not currently used as a criterion for PDAC diagnosis, recent investigations indicated that they may be promising biomarkers in the near future.

Circulating tumor cells (CTCs) are tumor cells that have acquired the ability to enter the circulatory system. Studies have reported the presence of CTCs in peripheral blood in 40%-100% of pancreatic cancer patients[76,77], and their potential as biomarkers of PDAC was demonstrated recently[76-83]. CTCs have the potential to provide a surrogate for "real-time biopsy" of tumor biological activity. However, as CTCs are extremely rare, both enrichment and sensitive methods of detection are required for their enumeration[79]. Recently, using a modular system with innovative features, EpCAM positive CTCs were isolated from PDAC patients at high purity (> 86%) and with excellent yields (mean = 53 CTCs per mL)[76]. However, the high cost and involved procedure associated with this system constitute an obstacle to its clinical application.

CONCLUSION

The early detection of pancreatic cancer may benefit patients with PDAC. The slow development and progression of pancreatic cancer are closely associated with the activation of oncogenes, inactivation of tumor suppressor genes, altered expression of miRNAs, and activated tumor microenvironment. Therefore, a better understanding of the pathogenesis of PDAC may help detect PDAC or PDAC precursors at the early stages of the disease. However, a low rate of PDAC incidence and the limitations of current screening strategies make early detection difficult. A cost-effective screening strategy is required. The association of DM with PDAC may provide clues for early diagnosis and assessment of the progression of PDAC. Advances in the identification of novel circulating biomarkers including serological signatures, autoantibodies, epigenetic markers, circulating tumor cells and miRNAs have provided potential tools for the early detection of PDAC. However, there are currently no prospective studies investigating screening methods for PDAC in patients with new-onset DM, and biomarkers

useful for this purpose or their combinations remain unidentified. Therefore, further studies are required in this field of research.

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2014 ADVANCES IN PANCREATIC CANCER

Nuclear receptors and pathogenesis of pancreatic cancer

Simone Polvani, Mirko Tarocchi, Sara Tempesti, Andrea Galli

Simone Polvani, Mirko Tarocchi, Sara Tempesti, Andrea Galli, Department of Experimental and Clinical Biomedical Sciences, University of Florence, 50134 Florence, Italy
Author contributions: Polvani S made the literature review and wrote the manuscript; Tempesti S and Tarocchi M critically revised the manuscript; Galli A supervised the manuscript; all authors approved the final version of the manuscript.
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Correspondence to: Andrea Galli, Professor, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Viale Pieraccini 6, 50134 Florence, Italy. a.galli@dfc.unifi.it
Telephone: +39-55-4271419   Fax: +39-55-4271297
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of their role are not fully understood. The purpose of this review is to highlight the striking connections that link peroxisome proliferator activated receptors, retinoic acid receptors, retinoid X receptor, androgen receptor, estrogen receptors and the orphan NR Nur, chicken ovalbumin upstream promoter transcription factor Ⅱ and the liver receptor homologue-1 receptor to PDAC development, connections that could lead to the identification of novel therapies for this disease.

Core tip: Pancreatic cancer is a devastating disease with well defined genetic alterations made deadly by its subtle nature and the lack of effective drugs. Nuclear receptors (NR) are ligand-regulated transcription factors involved in important cellular functions and tempting targets for drug development. There are now evidences that classical ligand-activated peroxisome proliferator activated receptors, retinoic acid receptors, retinoid X receptors, androgen receptor, estrogen receptors and orphan Nur, chicken ovalbumin upstream promoter transcription factor Ⅱ and liver receptor homologue-1 NR are involved in the pathogenesis of pancreatic cancer. No clinical application of these NR in pancreatic cancer cure is reported but a more comprehensive analysis of NR action could lead to the identification of new treatments for this disease.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with a median overall survival time of 5 mo and the five years survival less than 5%, a rate essentially unchanged over the course of the years. A well defined progression model of accumulation of genetic alterations ranging from single point mutations to gross chromosomal abnormalities has been introduced to describe the origin of this disease. However, due to the its subtle nature and concurring events PDAC cure remains elusive. Nuclear receptors (NR) are members of a large superfamily of evolutionarily conserved ligand-regulated DNA-binding transcription factors functionally involved in important cellular functions ranging from regulation of metabolism, to growth and development. Given the nature of their ligands, NR are very tempting drug targets and their pharmacological modulation has been widely exploited for the treatment of metabolic and inflammatory diseases. There are now clear evidences that both classical ligand-activated and orphan NR are involved in the pathogenesis of PDAC from its very early stages; nonetheless many aspects

INTRODUCTION

The most frequent form of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) one of the most lethal cancer and the fifth cause of cancer death in the Developed Countries[1]. Treatment of PDAC is primarily a combination of curative surgery and adjuvant chemotherapy with modestly effective drugs[2]. Unfortunately, due to absence of specific symptoms, a high percentage of patients at the time of diagnosis present a incurable locally advanced or metastatic disease that precludes a successful surgical resection, the only possible curative methods for PDAC, at least for early stages diseases.

Approved in 1996[2], Gemcitabine is the frontline standard chemotherapy used essentially in monotherapy for the treatment of pancreatic cancer with modest results; the innate or acquired resistance to chemotherapy drugs of PDAC remains the major obstacle to its successful control[3]. Early detection of PDAC is difficult if not impossible: benign and malignant lesions share similar clinical presentations and imaging features, making imaging detection of early disease difficult[4], and the majority of available molecular markers possess low specificity[5]. Therefore the median overall survival time is 5 mo and the five years survival is less than 5%, a rate essentially unchanged over the course of the years[6]. Consequently, a deeper understanding the pathobiology of this disease is essential to lead to new targeting strategies.

The route to PDAC

Despite the effort of the scientific community, the etiology of PDAC is still poorly understood undermining the efforts for its prevention and cure. A large number of epidemiological studies suggested that tobacco smoking, alcohol consumptions, obesity, chronic pancreatitis, genetic risk factors and diabetes are risk factors of PDAC[7-9].

Pancreatic adenocarcinoma arises from three precursor lesions: the microscopic pancreatic intraepithelial neoplasia (PanIN) and the macroscopic intraductal papillary mucinous neoplasm and mucinous cystic neoplasm (Figure 1)[10,11].

PanINs are the more frequent preneoplastic precursors of PDAC[12]; they are classified according to the accumulation of architectural, cytologic, and genetic alterations: from PanIN 1 with the appearance of columnar cells with mucin, to PanIN3 (also called carcinoma *in situ*) characterized by a severe cyto-architectural athypia[10].

PDAC is a disease with a well defined progression model of accumulation of genetic alterations ranging from single point mutations to gross chromosomal abnormalities[13-17]. The most frequent and studied alterations determine the activation of epidermal growth factor receptor-KRAS pathway[15]. Although almost all patients posses at least one of these mutations, the late stage disease is characterized by increased genome instability and heterogeneity with an average of 63 genetic alterations, the majority of which are point mutations, grouped in a core set of 12 cellular signaling pathways[18].
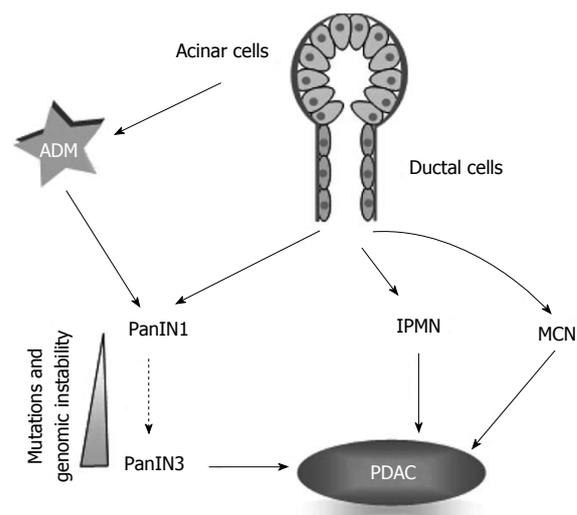
Figure 1 Origin of pancreatic ductal adenocarcinoma. It is widely accepted that pancreatic ductal carcinoma (PDAC) arises from precursor lesions derived from ductal cells; however, recently another model has been proposed where PDAC arises from acinar cells through a process called "acinar to ductal metaplasia" (ADM). PanIN: Intraepithelial neoplasm; IPMN: Intraductal papillary mucinous neoplasm; MCN: Mucinous cystic neoplasm.

Morphological progression from PanIN to PDAC is paralleled by the accumulation of these genetic alterations in a progression model resembling the colon cancer model. Ductal origin of PanIN and PDAC is however questioned and a new model has been proposed where the cancer ductal cells in PanIN lesions originate from metaplastic acinar cells in a process called "acinar to ductal metaplasia" (ADM)[19].

Nuclear receptors: Classification, structural features, and ligands

Nuclear receptors (NRs) are members of a large superfamily of evolutionarily related ligand-regulated DNA-binding transcription factors present in most metazoan[20].

The first NR was only cloned in the '80s by Professor Evans R[21] long after the presence of NR was detected biochemically[22,23]; so far 48 NR has been identified in human by genome sequencing. All NR share characteristic structural features or domains named A to F from the N-terminal to the C-terminal; however, defining features of NR are only the presence of two highly conserved regions: the DNA binding domain (DBD) and the ligand binding domain (LBD), that can function independently[24] and are located in the region C and in the region E of the protein, respectively (Figure 2).

NR are classified into six different subfamilies on homology basis: NR1 (thyroid hormone like), NR2 (HNF4-like), NR3 (estrogen like), NR4 (nerve growth factor IB-like), NR5 (fushi tarazu-F1 like), and NR6 (germ cell nuclear factor like), all originally named from the first member identified. A seventh subclass, NR0, has been introduced to classify two receptors, DAX-1 and SHP, that do not possess the DBD[25] (Table 1, information on ligands obtained from the "nuclear recep-
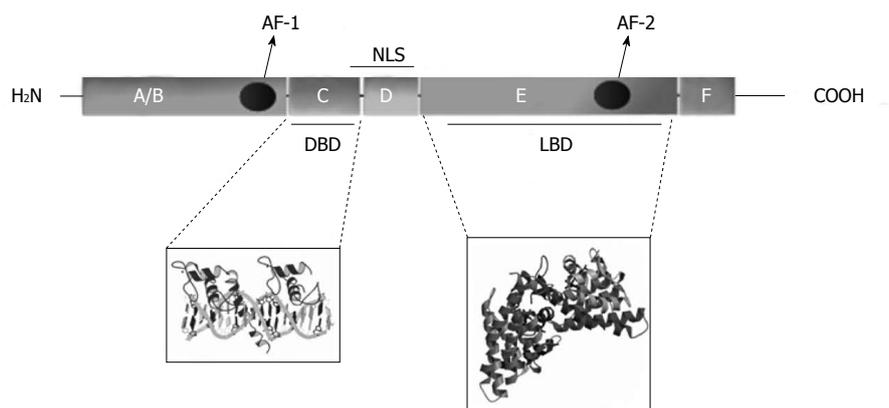
Figure 2 Domains and structural features of a classical nuclear receptor. A typical nuclear receptor (NR) consists of 6 region (A to F); region F may or may not be present. Region D (hinge) contains the nuclear localization signal (NLS); other NLSs may be present in region E. AF-1: Activator function 1; AF-2: Activator function 2.
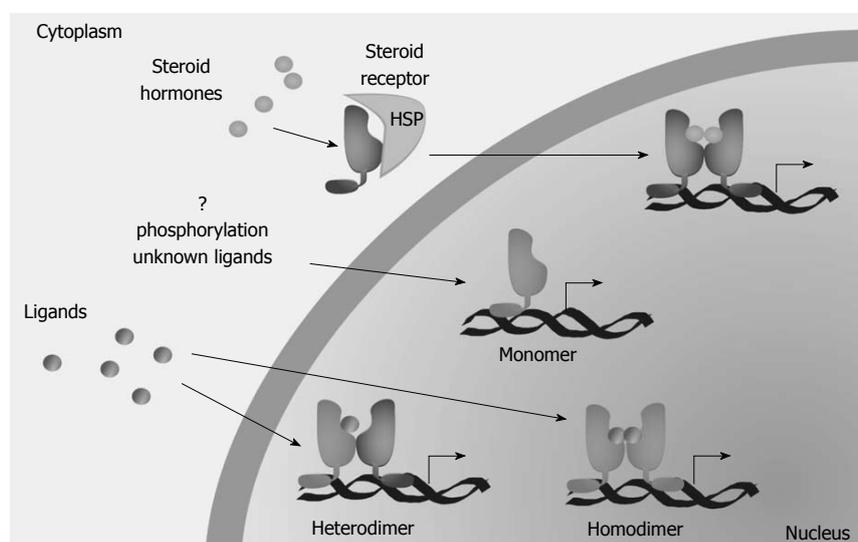
Figure 3 Mechanisms of action of nuclear receptors. Type Ⅰ nuclear receptors (NR) (steroid receptors) are complexed with heat shock proteins (HSP) and maintained in the cytoplasm in the absence of ligands. The other receptors are instead mainly nuclear and the ligands induce hetero- (for type Ⅱ receptors) or homo-dimerization (for type Ⅲ receptors). Furthermore, a group of receptors (type Ⅳ) whose regulation is poorly known act as monomers.

tor signaling atlas", NURSA, www.nursa.org).

NR ligands are small hydrophobic molecules that bind to the LBD; retinoids, fatty acids, cholesterol, lipophilic hormones and vitamins, as well as some antibiotics, xenobiotics and synthetic drugs are all NR ligands. The ligand binding induces a conformational change that modify the DBD ability to bind specific DNA sequences called response elements. NR act as monomeres, homodimers or heterodimers with the retinoid-X-receptor (RXR) (Figure 3). Upon DNA binding, the final transcriptional activity depends on the presence of co-activator or co-repressor molecules[26].

NR physiologic functions vary from the regulation of metabolism, to growth and development; NR are also implicated with a number of diseases such as cancer. The association of NR with major diseases has transformed these proteins in the most popular and promising drug targets thanks also the properties of their ligands that can easily cross the cell membrane[27].

NR IN PANCREATIC CANCER

NRs are important in the development and homeostasis of the pancreas and their role in PDAC development is the subject of intense study by the scientific community. Here, we will describe the role of a group of these receptors, specifically the peroxisome proliferator activated receptors, the retinoid receptors, the androgen and estrogen receptors, and the orphan NRs.

PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS

The peroxisome proliferator activated receptors (PPARs) belong to the NR1 (thyroid-like) subfamily of NR. Three PPARs are known: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3). PPARγ is the only PPAR with three isoforms with different spatial distribution[28]. They were identified as NR that responded to peroxisome

Table 1 Nomenclature of nuclear receptors					
Subfamily official name (class)	**NRNC group**	**Member trivial name**	**Official name**	**Abbreviation**	**Ligand**
NR0 (domain-depleted receptors)	B [DAX-like receptors (DAX, SHP)]	Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1	NR0B1	DAX-1	Orphan
NR1 (TR/RAR/PPAR/ VDR-like receptors)	A (thyroid hormone receptors)	Short heterodimer partner	NR0B2	SHP	Orphan
		Thyroid hormone receptor α	NR1A1	TRα	GC-1, thyroid hormone
	B (retinoic acid receptors)	Thyroid hormone receptor β	NR1A2	TRβ	GC-1, thyroid hormone
		Retinoic acid receptor α	NR1B1	RARα	Am 580, all-trans-Retinoic acid, Arotinoid acid
		Retinoic acid receptor β	NR1B2	RARβ	Am 580, all-trans-Retinoic acid, Arotinoid acid
	C (peroxisome proliferator activated receptors)	Retinoic acid receptor γ	NR1B3	RARγ	Am 580, all-trans-Retinoic acid, Arotinoid acid
		Peroxisome proliferator-activated receptor α	NR1C1	PPARα	GW409544, GW7647, GW6471, Pirinixic acid, Palmitic acid, Leukotriene B4
		Peroxisome proliferator-activated receptor β	NR1C2	PPARβ/δ	Eicosapentaenoic acid, GW0742
		Peroxisome proliferator-activated Receptor γ	NR1C3	PPARγ	Rosiglitazone, GW1929, GW9662, GW409544, GW7647, 15-Deoxy-Δ-12,14-prostaglandin, 15-Deoxy-Δ-12,14-PGJ2
	D [Rev-Erb (NRD, E75)]	Rev-erbα	NR1D1	Rev-erbα	Orphan
	F (RAR-related orphan receptors (ROR, HR3)		NR1D2	Rev-erbβ	Orphan
		Retinoic acid receptor-related orphan receptor α	NR1F1	RORα	Melatonin, CGP 52608
		Retinoic acid receptor-related orphan receptor β	NR1F2	RORβ	Melatonin, CGP 52608
		Retinoic acid receptor-related orphan receptor γ	NR1F3	RORγ	Melatonin,CGP52608
		Liver X receptor β	NR1H2	LXRβ	GW3969, T0901317, oxysterols
	H (ecdysone-like receptors)	Liver X receptor α	NR1H3	LXRα	GW3969, T0901317, oxysterols
		Farnesoid X receptor α	NR1H4	FXRα	GW4064, bile acid chenodeoxycholic acid
	I (vitamin D3-like receptors)	Vitamin D3 receptor	NR1I1	VDR	1,25-dihydroxyvitamin D3
		Pregnane X receptor	NR1I2	PXR	Hyperforin, SR12813, rifampicin, pregnenolone carbonitrile, T0901317, 24(S),25-epoxycholesterol, butamben
		Constitutive androstane receptor	NR1I3	CAR	Androstanol, CITCO, phenobarbital, ATE
NR2 (HNF4/RXR/ TLL/COUP-like receptors)	A (hepatocyte nuclear factor 4)	Hepatocyte nuclear factor 4 α	NR2A1	HNF4α	Palmitoyl coenzyme A
		Hepatocyte nuclear factor 4 γ	NR2A2	HNF4γ	Orphan
	B (retinoid X receptors)	Retinoid x receptor α	NR2B1	RXRα	LGD 100268, GW0791, 9-retinoic acid
		Retinoid x receptor β	NR2B2	RXRβ	LGD 100268
		Retinoid x receptor γ	NR2B3	RXRγ	LGD 100268
		Testis receptor	NR2C1	TR2	Orphan
	E (tailless-like receptors)		NR2C2	TR4	Orphan
		Tailless	NR2E2	TLL	Orphan
		Photoreceptor-specific nuclear receptor	NR2E3	PNR	Orphan

	F [COUP-TF-like receptors (COUP-TF, SVP, EAR2)]	Chicken ovalbumin upstream promoter transcription factor Ⅰ	NR2F1	COUP-TFI	Orphan
		Chicken ovalbumin upstream promoter transcription factor Ⅱ	NR2F2	COUP-TFⅡ	Orphan
		ErbA2-related gene-2	NR2F6	EAR2	Orphan
NR3 (ER/ERR/GR/MR/PR/AR)	A	Estrogen receptor	NR3A1	Erα	Fulvestrant, 17β-estradiol, 4-hydroxytamoxifen, Raloxifene
			NR3A2	Erβ	Fulvestrant, 17β-estradiol, 4-hydroxytamoxifen, Raloxifene
	B	Estrogen receptor-related receptor	NR3B1	ERRα	Orphan
			NR3B2	ERRβ	GSK4716, Diethylstilbestrol
			NR3B3	ERRγ	GSK4716, 4-hydroxytamoxifen
	C	Glucocorticoid receptor	NR3C1	GR	Dexamethasone, hydrocortisone, RU486
		Mineralocorticoid receptor	NR3C2	MR	Spironolactone, aldosterone, RU486
		Progesteron receptor	NR3C3	PR	R5020, progesterone, RU486
		Androgen receptor	NR3C4	AR	Dihydrotestosterone, RU486, Bicalutamide, R1881
NR4 (NGFIB-like receptors)	A [nerve growth factor IB-like receptors (NGFIB, NURR, NOR, HR38, CNR-8)]	Growth factor-inducible immediate early gene Nur77	NR4A1	Nur77	Orphan
		Nur-related protein 1	NR4A2	NURR1	Orphan
NR5 (FTZ-F1/SF1-like receptors)	A [fushi tarazu F1-like receptors (SF1, FTF, FTZ-F1)]	Neuron-derived orphan receptor 1	NR4A3	NOR1	Orphan
		Steroidogenic factor-1/ELP	NR5A1	SF1	Orphan
NR6 (GCNF)	A (germ cell nuclear factor)	Liver receptor homolog 1	NR5A2	LRH-1	Orphan
		Germ cell nuclear factor 1	NR6A1	GCNF1	Orphan

PPAR: Peroxisome proliferator-activated receptor; PDAC: Pancreatic ductal carcinoma; TZD: Thiazolidinediones; IFN: Interferon; RAR: Retinoic acid receptor; RXR: Retinoid X receptor; AR: Androgen receptor; ER: Estrogen receptor; LHR-1: Liver receptor homologue-1 receptor; COUP-TFⅡ: Chicken ovalbumin upstream promoter transcription factor Ⅱ.

proliferators, heterogenous chemicals that increase the number of peroxisomes (making them "proliferate") in hepatocytes[29]. Natural ligands for PPARs are free fatty acids and PPARγ is also activated by 15-Deoxy-delta (12,14)-prostaglandin J(02)(15d-PGJ2)[30]. Some other PPAR ligands are the PPARα agonists hypolipidemic drugs fibrates and leukotriene B4 (LB4), the PPARγ specific agonist and antidiabetic drugs thiazolidinediones (TZD), and the PPARβ/δ specific agonist GW501516[31].

PPARs show a different expression pattern, and PPARβ is the most widely expressed[32]; they act as heterodimers with RXR and regulate complex gene networks especially in energy homeostasis and inflammation[28,33,34]. Furthermore they are involved in a spectrum of disease such as alcoholic liver disease and may mediates oxidative stress response[28,30,32,34].

PPARα
PPARα sustained activation leads to the development of cancers in the liver, testis and pancreas in rodents[35,36]. Moreover, PDX-1, an oncogene for pancreatic cancer that is overexpressed in PDAC[37], is a PPARα -dependent gene and its expression is downregulated by MK886, a specific PPARα antagonist[38]. However PPARα -dependent carcinogenesis has been recently questioned[39].

PPARβ/δ
Recently it has been reported that PPAR signaling, especially PPARβ/δ, is reduced in pancreatic cancer relapse, compared to primitive cancer[40], but PPARβ/δ has been suggested to be a critical component of the angiogenetic switch in pancreatic cancer[41,42]. Abdollahi[42] showed that the expression of PPARβ/δ detected by immunohistochemistry in human pancreatic specimens highdensity tissue microarrays correlated with tumor staging; indeed PPARβ/δ staining intensity increased from normal pancreas to chronic pancreatitis, pancreatic cancer and metastasis and the up-regulation of PPARβ/δ is actually more enhanced in the tumor vasculature and in the tumor stroma[42]. High expression of PPARβ/δ in tumor is also confirmed by a recent paper[31]. Elevated PPARβ/δ expression levels are also highly correlated with advanced stages of tumor progression and with increased risk for tumor recurrence or distant metastasis[42]
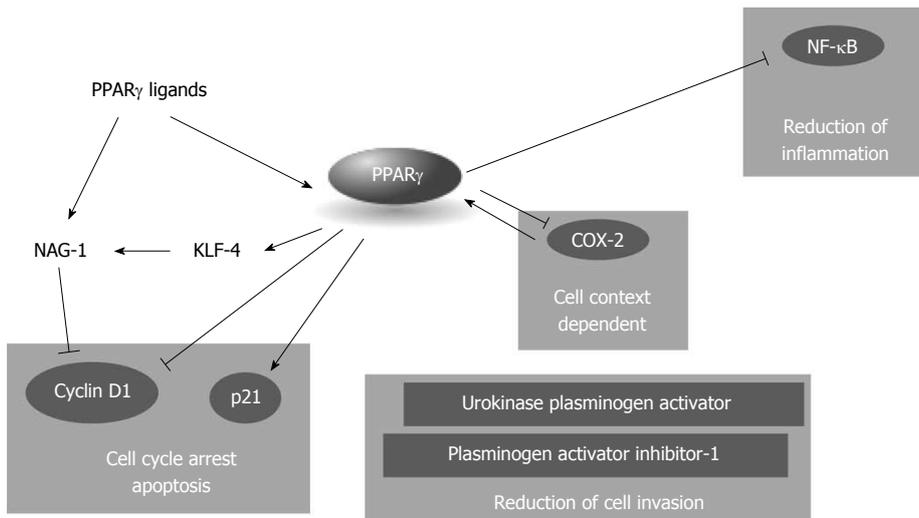
Figure 4 Peroxisome proliferator activated receptors and pancreatic ductal carcinoma. Peroxisome proliferator activated receptor (PPAR)γ acts as a tumor inhibitor at multiple levels, blocking cell cycle progression, inflammation, and cell invasion. NAG-1: Non steroidal anti-inflammatory drug-activated gene-1; Cox-2: Ciclooxigenase-2; NF-κB: Nuclear factor-κB.

and PPARβ/δ has been proposed as a "central hub" in tumor angiogenesis given that tumor growth and angiogenesis were greatly reduced when tumor cells were implanted in PPARβ/δ null mice[42].

PPARβ/δ is also expressed in human pancreatic cancer cells and its activation regulates the metallo-protease matrix metalloproteinases (MMP)-9, decreasing cancer cells ability to transverse the basement membrane[31]. PPARβ/δ activation reduces the tumor necrosis factor (TNF)α-induced expression of various genes implicated in metastasis increasing the availability of the transcriptional repressor B-cell lymphoma (BCL)-6. BCL-6 is bound to PPARβ/δ and is released after GW501516 treatment resulting in decreased MMP-9 expression. These results suggest that increased expression of PPARβ/δ regulates pancreatic cancer cell invasion sequestering BCL-6 and hence inducing MMP-9 mediated invasion[31].

PPARγ

PPARγ is not only implicated in adipocyte differentiation, lipid accumulation, and glucose homeostasis but it is also an important regulator of inflammation *via* the inhibition of, or the interference with, proinflammatory signalings such as signal transducers and activators of transcription (STATs), nuclear factor-κB (NF-κB), and activator protein-1 (AP-1)[28,30].

PPARγ is expressed in primary PDAC[43-46] and strongly correlates with a more advanced clinical stage; PPARγ staining is also associated with shorter overall survival and its has proved to be an independent prognostic factor in uni- and multi-variates analysis[43,45]. However, whereas Kristiansen *et al*[45] found a strong overexpression of PPARγ by expression profiling in 19 microdissected carcinoma compared to 14 ductal epithelia, Pazienza *et al*[44] did not find significant alterations in the expression levels of PPARγ in pancreatic cancer. A possible explanation of this discrepancy may be that is the expression *"per*

se" of PPARγ, and not its levels, important in pancreatic cancer progression. Interestingly, a large number of studies have demonstrated that TZD reduce the risk of PDAC[9].

A genetic association PPARγ/PDAC has been tested by two different groups[47,48] analyzing the expression of the single nucleotide polymorphisms (SNP) Pro12Ala that has been associated with reduced risk of diabetes and some cancers[47]. Fesinmeyer *et al*[48] demonstrated that this SNP is associated with increased risk of PDAC in a high-risk sample of smokers randomized to high-dose vitamin A; however, two years later, a similar study in obese and diabetic patients demonstrated a protective role of the SNP, prompting the need of further studies[47].

In vitro, the role of PPARγ is controversial but it is generally accepted that the receptor acts as a tumor inhibitor at multiple levels (Figure 4). PPARγ is expressed in human pancreatic cancer cell lines[46,49-55] and its expression follows a circadian rhythmicity with a period of 24 h that could potentially influence the cell phenotype and the human disease behavior[55]. Agonists of PPARγ such as TZD, its natural ligand 15d-PGJ2, or 1,1-Bis(3-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs), induce cell cycle arrest in G1, apoptosis and ductal differentiation in pancreatic cancer cells[46,53,54,56-58]. However some of the effects associated with PPARγ activation are instead receptor independent: this is especially true for TZD agonists whose receptor independent effects have been widely described[51,52,59-69].

Cell cycle arrest is associated to decreased PPARγ-dependent expression of cyclin D1[53,54,57,58] whereas the reported induction of p21 might be PPARγ-dependent or PPARγ-independent[52,54]. Deletion analysis of the p21 promoter indicates that PPARγ-dependent activation of p21 requires GC-rich sites in the proximal region of the promoter[54]. It is worth to note that some agonists of PPARγ[61] may induce a PPARγ independent down-

regulation of cyclin D1, due to induction of non steroidal anti-inflammatory drug-activated gene-1 (NAG-1). NAG-1 is a member of the TGF-β superfamily involved in tumor progression acting as a pro-apoptotic gene. Interestingly, it has been reported that NAG-1 expression is positively regulated by PPARγ: MCC-555, a PPARγ agonist, induces the expression of the transcription factor KLF-4 in PPARγ-dependent manner who subsequently enhances the NAG-1 promoter activity[51,52]. PPARγ agonists reduce the invasive capacity of PDAC cells with a PPARγ dependent mechanism[50]. The PPARγ ligands 15d-PGJ2 and ciglitazone attenuate pancreatic cancer cell invasion increasing plasminogen activator inhibitor-1 and decreasing urokinase plasminogen activator levels resulting in the reduction of total urokinase activity in pancreatic cancer cells[50]. Interestingly, the PPARγ antagonist T0070907 suppresses pancreatic cancer cell motility by altering the localization of p120 catenin and by suppressing the activity of the Ras-homologous GTPases Rac1 and Cdc42[49].

The effects of PPARγ activation or inhibition by specific molecules sinergize or interact with other pathways or other NR activations[53,56,62-64]. Combination of recombinant interferon-β (IFN-β) and the PPARγ agonist troglitazone induces a synergistic effect on the growth inhibition of pancreatic cancer cells, through the counteraction of the IFN-β-induced activation of STAT-3, MAPK and AKT and the increase in the binding of both STAT-1 related complexes and PPARγ with specific DNA responsive elements. The combination induces also an increase in autophagy and a decrease in anti-autophagic bcl-2/beclin-1 complex formation, mediated by the inactivation of the AKT/mTOR-dependent pathway[64]. PPARγ form mandatory heterodimers with RXRα and its activity is maximal in the presence of RXRα agonists; it is not surprising then that co-treatment with PPARγ and RXRα agonists exacerbates the effects of PPARγ increasing the inhibition of cell growth[53,62,63]. Synergistic effects on growth inhibition are also visible when inhibitors of ciclooxigenase-2 (Cox-2) and PPARγ agonists are used in combination[56,65]. Cox-2 is an inducible ciclooxygenase that contributes to the metabolism of arachidonic acid forming prostaglandin H2, a precursor of 15d-PGJ2[66]. Cox-2 is a downtarget of PPARγ and its expression may be either induced or repressed by the NR, depending on the cell context[30]. However, selective Cox-2 inhibitors have opposite effects in pancreatic cancer depending on Cox-2 expression: in high Cox-2-expressing cells the inhibitors reduced tumor growth; conversely, in Cox-2 negative or low expressing cancer cells the inhibitors, at very high concentrations, enhance tumor progression increasing intratumoral VEGF and tumor angiogenesis in a PPARγ-dependent way[65].

PPARγ is known to reduce tumor growth in mice *in vivo*[49,62,65,67] and its activation increases the gemcitabine mediated tumor suppression[68]. Tumor growth inhibition mediated by PPARγ may be due to reduced inflammation and increased activation of anti-inflammatory genes[30,67].

Genetic deletion of Ikk2, a component of the canonical NF-κB signaling pathway, in the Kras(G12D)Pdx1-cre mouse model of pancreatic cancer, substantially delays pancreatic oncogenesis and results in downregulation of the classical Notch target genes Hes1 and Hey1[67]; in the same model TNF-α stimulation resulted in increased Hes1 expression and consequent suppression of PPARγ expression facilitating the formation of a inflammatory pro-tumoral enviroment; induction of PPARγ instead may block NF-κB induced processes[30] reducing or delaying tumor formation[67].

Despite all these intriguing discoveries on PPARγ role in PDAC, clinical application of PPARγ modulation has recently suffered yet another failure when a new oral anticancer agent with LB4 antagonist and PPARγ agonist properties[69,70], the LY29311, did not demonstrate any benefit in association with gemcitabine in unpretreated patients with advanced PDAC[69].

RETINOIC AND RETINOID RECEPTORS

Retinoic acid receptors (RARs) and RXRs are NRs transcription factors that bind retinoids, natural and synthetic molecules structurally and/or functionally related to vitamin A, and regulate cell differentiation, proliferation, and survival[25,71,72]. A list of retinoids with biological functions comprises, but is not limited to, all trans retinoic acid (atRA), 9-*cis*-retinoic acid (9-*cis*-RA), 11-*cis*-RA, 13-*cis*-RA, being the atRA the predominant physiological form; retinoids that specifically bind to RXR are called rexinoids and have been effective in cancer treatment. RARs can be activated by both atRA and 9-*cis* RA, while RXRs are exclusively activated by 9-*cis*-RA, initially identified as a *bona fide* RXR ligand *in vitro*[71], but never detected *in vivo*[73]. Other RXR natural ligands have been identified *in vivo* but they are not RXR specific ligands[74-76].

RARs and RXRs are each encoded by three different genes that give rise to the -α, -β, -γ isoforms of RXR and RAR, each presenting transcription variants, and characterized by different spatial distribution[77,78]. RXRs were identified as cofactor for efficient binding of RAR to its DNA response elements[79], but unique among the NRs, RXR play a modulatory role along multiple pathways forming mandatory dimers with thyroid hormone receptor, PPAR, vitamin D receptor (VDR), RAR, Nur77, *etc*.[25,71]; in these heterodimers RXR may function as an active partner (such in the case of PPARγ:RXR dimers) meaning that the dimers respond to 9-*cis*-RA, or as silent partner and the dimers do not respond to RA.

Due to their regulatory potential, these NRs are major drug targets for a number of pathologies, including cancer and metabolic diseases.

RAR and RXR receptors are expressed during pancreatic organogenesis and are essential for ductal differentiation[80,81]. Retinoid receptors are more expressed in the exocrine compartment, usually during late gestation, with a strong lineage specificity. Exogenous 9-cis-

RA induces predominantly ducts instead of acini, plus more mature endocrine architecture, whereas exogenous atRA induces predominantly acini instead of ducts, with no apparent endocrine effect[80]. RAR-selective agonists mimicked the acinar suppressive effect of 9-*cis*-RA, suggesting that RAR-RXR heterodimers are critical to ductal differentiation; however, retinoids do not regulate exocrine lineage selection cell-autonomously but epithelial-mesenchymal interactions are mandatory given that 9-*cis*-RA does not induce ductal differentiation in the absence of mesenchyme and requires the presence of laminin-1[81]. The ability to restore a more differentiated phenotype and to regulates ductal differentiation may explain the effects of retinoids.

Expression of RXR and RAR has been described in pancreatic cancer cell lines and PDAC[42,53,62,63,82-88] but their biological and clinical significance is not clear: in most cases RXR and RAR receptors apparently act as tumor suppressors in PDAC cancer both *in vivo* and *in vitro*[43,53,62,63,84-88], inducing arrest in cell proliferation and differentiation although results suggestive of a pro-oncogenic role are also reported[89-91].

A differential expression of RAR-α, -β, and -γ, and RXRα was detected in histological sections of human PDAC and their adjacent normal tissues. Whereas all four receptors were detected in adjacent normal pancreatic tissue specimens, RARβ mRNA transcripts were detected in only 67% of the malignant tissues and when expressed, the level of expression was significantly lower than that of the corresponding adjacent normal tissues, especially in moderately- and poorly-differentiated cancers[92]; these results are in agreement with previous papers showing that RARβ expression is lost during PDAC malignant transformation[84]. The mechanisms at the basis of RARβ mRNA downexpression are not known but it is worth noting that in pancreatic endocrine neoplams the NR promoter is often hypermethylated[93,94], suggesting that in certain pancreatic carcinomas the reduction or loss of RARβ expression by epigenetic mechanisms might be associated with the development or progression of tumors[92]; interestingly one missense mutation in RARβ has also been identified in PDAC[18]. The anti-tumoral role of RARβ is confirmed by its overexpression in DAN-G pancreatic cancer cells that results in induction of differentiation and inhibition of proliferation *in vivo* and *in vitro*[84].

Immunohistochemical evaluation of PPARγ and RXRα protein expression in 65 PDAC patients statistically analyzed in relation to clinicopathological characteristics, tumor proliferative capacity, and patients' survival showed that 75% of patients tested positive for PPARγ and 85% stained positive for RXRα. Interestingly, RXRα positivity was significantly associated with tumor proliferative capacity and PPARγ positivity but RXRα failed to predict patients' survival[43].

In vitro, RXR and RAR involvement in PDAC has been usually tested by means of specific agonists or antagonists. Retinoids may be useful agents for the treatment of pancreatic cancer; however, RAR-selective retinoids produce unwanted side effects. In contrast, RXR-selective retinoids produce fewer side effects. The reported results indicate that these receptors often possess antiproliferative and pro-differentiative effects whereas reports on induction of apoptosis are mixed[82,83,88].

In 13 cell lines established from patients who underwent surgery for PDAC Albrechtsson *et al*[86] detected the expression of the RAR and RXR subtypes and evaluated the effect of atRA and 9-*cis*-RA on cell proliferation. They demonstrated that RARα, β and RXRβ were expressed in most of the cell line. RXRγ was expressed in about half of them and RARγ in only one whereas the RXRα receptor was expressed in all cell lines. Incubation of the cells with atRA or 9-*cis*-RA reduced cell proliferation, although only about half of the cell lines responded to the latter[86]. These results partially contradict a previous paper that showed that pancreatic cancer cell lines *in vitro* responded to 9-*cis*-RA but not atRA at clinically relevant concentrations[87]. Moreover, as previously reported[53], 9-*cis*-RA acts additively with the TZD Troglitazone blocking the cells in G1 phase through reduction of cyclin D1 levels.

The RXR-selective retinoid, AGN194204 inhibits the proliferation of pancreatic cancer cells more efficiently than RAR-selective retinoids, but does not increase the apoptosis, whereas other retinoids are also able to induce apoptosis[82]. Block of cell proliferation in these cells is associated with reduced cyclin E and cyclin dependent kinase 6 levels, an effect reversed by the RXR antagonist AGN195393 but not by RAR antagonist AGN193109. Treatment of MIAPaCa-2 cells with AGN194204 and cytotoxic agents such as gemcitabine, 5-fluorouracil, or IFNγ resulted in an additive but not synergistic reduction in cell number[95]. Interestingly, the retinoid-related ligand AGN193198 reduces BxPC-3, MIAPaCa-2 and AsPC-1 cell proliferation (blocking the cell in the S phase) more efficiently than high-affinity RAR- or RXR-selective retinoids and induces apotosis[88]; however the compound does not activate transcription from RAR or RXR response elements and its effects on cell survival are not reversed by treatment with RAR- or RXR receptor-selective antagonists. These results suggest that AGN193198 (but we may not exclude also other retinoids) might act independently of the classical retinoid receptors.

Treatment of pancreatic cancer cells with 9-*cis*-RA induces apoptosis lowering the ratio Bcl2/Bax2 and requires the presence of RARγ[83]. Interestingly, 9-*cis*-RA acting on RXRα may induce the nuclear export of Nur77[96,97], facilitating its interaction with Bcl2 and hence increasing the apoptosis (see later for details).

Retinoids possess the ability to induce pancreatic cancer differentiation *in vitro*[82,89,98]. The differentiation phenotype changes are associated with increase in aerobic metabolism, expression of mucins, synthesis and secretion of TGF-β, and reduction of EGF receptor expression[82]. This differentiation effects are dependent

on TGF-β, because co-treatment with atRA and a pan-TGF-β neutralizing antibody abolishes the anti-prolif-erative and pro-differentiative effect of the retinoid and reduces MUC4 expression[82,89]. As previously described, ADM might play an important role in PDAC develop-ment and DSL-6A/C1 cells, who expresses RAR-α and -β and RXRα, represent an *in vitro* model of this car-cinogenic sequence[98]. Treatment of DSL-6A/C1 cells with retinoids results in a time- and dose-dependent in-hibition of cell growth, paralleled by a retinoid-mediated transactivation of a pTK:betaRAREx2-luciferase report-er; growth inhibition is reverted by the RARα specific antagonist Ro 41-5253, suggesting that the RARα might influence ADM[98].

Regulation of expression of mucins (MUCs) by reti-noids however raises questions regarding the response of pancreatic tumor cell *in vivo*. RAR and RXR recep-tors have been reported to influence the expression of MUC4 and MUC17[89-91] and RXR:VDR response ele-ments are present in the promoter region of *MUC17* gene[90]. Both mucins are associated to the progression of pancreatic cancer: MUC17 is linked to the presence of lymph node metastasis[99] and MUC4 expression in-creases during progression of PDAC from PanIN1 to PanIN3, and it is highly expressed in invasive adenocarcino-mas[100-102]. The expression of *MUC17* gene is regulated by a 1146-bp DNA fragment upstream of MUC17 that contains GATA, NF-κB, Cdx-2 and RXR:VDR response elements, but no data are available on the role of the lat-ter. Instead, retinoids directly regulates the expression of MUC4[91], sinergistically with IFNγ and dependently of TGF-β. Interestingly, IFNγ has been shown to possess antitumor activity and it is well known that TGF-β pos-sess tumor suppressive and oncogenic activities[103]. At early stages TGF-β acts as a tumor suppressor, whereas at later stages tumor cells become resistant to its antip-roliferative effects but continue to secrete high quantity of the factor. Indeed, pancreatic tumors overexpress all the three TGF isoforms and this correlates with de-creased patient survival[104] and induction of epithelial to mesenchymal transition (EMT). On the other hand, although IFNγ is antiproliferative *in vitro* against pancre-atic cancer cells, the temporal aspect of this process has never been studied. Indeed, the expression of MUC4 does not require the continuing presence of IFNγ or RA which instead are required for the priming of MUC4 expression[91]. From a pathological point of view, aber-rant expression of mucins on the surface of PDAC cells may provide protection against the host's activated im-mune system while conferring antiadhesive properties upon the cells and hence favoring the EMT-mediated metastatization, casting a shadow on the use of retinoids *in vivo*. Nonetheless, retinoids have been used in phase Ⅱ clinical trials in the past[105-107] with mostly disappointing results. In 1998, basing on promising *in vitro* results, one trial in which patients with advanced PDAC were treated with 13-*cis*-RA and IFNα resulted in prolonged stable disease in two third of the patients[105]; this however con-

tradict a 1995 phase Ⅱ trial where the same therapeutic regimen did not improve patients condition[107]. Further-more, the combination of 13-*cis*-RA with gemcitabine in a more recent phase Ⅱ clinical trial, although well tolerated, did not determine an improvement in the re-sponse[106]. PDAC resistance to retinoid treatment might be dependent on the relative intracellular expression of the retinoids-binding proteins fatty acid-binding protein 5 (FABP5) and cellular retinoic acid-binding protein 2 (CRABP2)[108] that were shown to be critical for either antisurvival (CRABP2) or prosurvival (FABP5) effects of retinoic acid[109].

ANDROGEN AND ESTROGEN RECEPTORS

Androgen receptor (AR, NR3C4) and estrogen recep-tors (ER)-α and -β (NR3A1 and NR3A2) belong to the steroid receptor subfamily (NR3). These NR regulate multiple physiological processes including sexual devel-opment and are implicated in multiple cancers[110-113].

Their involvement in PDAC has long been suggested by the evidence that pancreatic cancer shows an appar-ent hormonal imbalance in the incidence with a male to female ratio ranging from 1.25-1.75:1[114,115], that ap-proach the 1:1 ratio with advancing age[116] (for recent reviews see[117,118]).

In the early '90s, the presence of AR in PDAC was ques-tioned, but recent papers clearly demonstrated that pancreat-ic cancer cells express detectable levels of AR[117,119,120]. *In vi-tro*, PDAC cancer cells variably respond to the treatment with the agonist testosterone, showing a modest increase in cell proliferation[119,121]. Flutamide, an AR blocker used for the treatment of prostate cancer, induces a reduction in cell proliferation that does not however correlate with AR expression levels[119]. Furthermore, flutamide treat-ment does not alter the cell response to gemcitabine *in vitro* and *in vivo*[119]. AR activity is modulated by IL-6, an inflammatory cytokine overexpressed in pancreatic can-cer[120,122]. IL-6 enhances STAT3 and MAPK pathways that in turn increase the AR transcriptional activity; IL-6 also enhances pancreatic cancer cell migration in the presence of AR, an effect blocked by the silencing of the receptor[120] (Figure 5A). In a double-blind placebo-controlled trial, flutamide doubled the survival duration when administered in a dosage of 250 mg three times daily[123]. This excellent result has not been confirmed by other small phase Ⅱ trials where flutamide was used in monotherapy or in combination with gemcitabine[124,125]. The lack of AR response in PDAC patients suggests that the tumor cells, although expressing the AR, are in a hormone-refractory proliferative status, as observed in prostate cancer[119].

The role of ER in PDAC is controversial: although several papers described the presence of ER (usually ERα) in primary PDAC, other reports did not detect the receptors at all[118,126,127] and the antiestrogen tamoxi-fen has been used in clinical trials with no benefit[118,128]. Expression of both ERα and -β has been described in
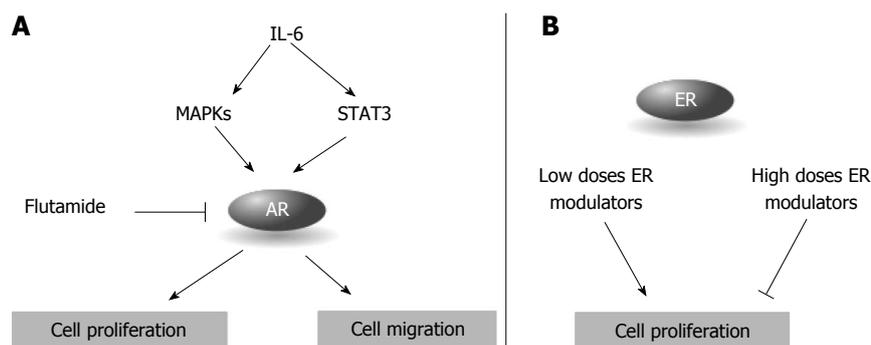
Figure 5 Steroid receptors in pancreatic ductal carcinoma. A: Androgen receptor (AR) regulates cell proliferation and migration and it is activated by the inflammatory cytokine interleukin-6 (IL-6); B: The effect of estrogen receptors (ER) on cell proliferation depends on the concentrations of the ER modulators.

pancreatic cancer cells[129] and a recent proteomic validation study in formalin-fixed paraffin-embedded tissues identified several proteins tightly associated through ERα[130]. *In vitro*, PDAC cells respond to the treatment with estrogens modulating agents: lower concentrations usually induce cell proliferation whereas high concentrations arrest cell proliferation (Figure 5B)[129]. The response seems dependent on the expression of ERα and ERβ, specifically to their ratio. ERα and ERβ share an almost perfect homology in the LBD that allows both of them to bind estrogen; other domains are instead less conserved with the most divergent region being the A/B domain, characterized by the absence of the activator function-1 in the ERβ. Analyzing the expression of the two estrogen receptors Iwao *et al*[129] found that pancreatic cancers showed significantly lower ERα mRNA levels than ER-negative breast cancers while ERβ mRNA levels (that were higher in ER-negative than ER-positive breast cancers) were significantly higher than ER-negative breast cancers; in seven out of eight pancreatic cancer cell lines ERβ outweighs ERα and cells with lower ERα/ERβ ratio tend to have higher responsiveness[128], suggesting that ERβ may play a more important role in PDAC[118,128]. Interestingly, phytoestrogens such as genistein block pancreatic cancer growth *in vitro* and show higher affinity for ERβ.

OTHER NRS: ORPHAN NRS IN THE SPOTLIGHT

Roughly half of the 48 human NR are classified as orphan NRs. The orphan NRs form a specific subgroup of the NR proteins characterized by different functional and evolutionary origin; orphan NR are distributed along the all the six NR subfamilies. These proteins have in common only the term "orphan receptor" conied few decades ago to describe, by definition, gene products that appear to belong to the nuclear receptor family on the basis of sequence identity but for whom no ligands is known[131,132]. Orphan receptors diverge also at the structural levels with various examples of members without all the classical features of nuclear receptor, such as the LBD or the DBD[132]. The discovery of the

orphan receptors drastically changed endocrinology introducing the "reverse endocrinology" approach where orphan receptors are used to identify new hormones and their associated biology, conversely to traditional endocrinology that identified hormones starting from their physiological or pathological effect[131].

Although the term "receptor" implies the existence of a natural ligand, this assumption is debated and not necessarily true for at least some of the orphan NRs[25]. In time, the number of orphan receptors has diminished due to the discovery of natural ligands for some of them that have then become "adopted", such in the case of PPARγ or FXR for example[25].

Here we will discuss recent findings on orphan receptors, specifically the NR4As, the liver receptor homologue-1 receptor (LRH-1) and the chicken ovalbumin upstream promoter transcription factor II (COUP-TF II) discussing their role in PDAC.

Nur77: A "two faces" action in pancreatic cancer

The orphan NR subfamily 4 subgroup A (NR4A) is comprised by three members: NR4A1 (also known as Nur77, testicular receptor TR3, or nerve growth factor 1b NGFI-B), NRA42 (Nurr-related factor 1) and NR4A3 (neuron-derived orphan receptor1, Nor-1)[25,133]. The first member of NR4A family was identified by differential hybridization in the rat pheochromocytoma cell line PC-12 cell as encoded by an immediate early gene (*i.e.*, a gene that is rapidly and transiently transcribed in response to stimuli) induced by the nerve growth factor[134]. As in the case of other NRs, the NR4A members show common features of a classic nuclear receptor and an high degree of sequence homology specifically in the DBD and LBD regions, where homology may be as high as 95% (Figure 6).

All three members are localized in the nucleus due to the presence of a nuclear localization signal in the DBD (three signals in the case of NR4A1). The three members of the family show a different and often overlapping expression in adult tissues, being Nur77 more abundantly and broadly expressed[133]. NR4A receptors might act as monomere or as homo- hetero-dimers with different affinity to DNA response elements; dimers show

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NR4A2   HVPMNPEPAGSHHVVDGQTFAVPNPIRKPAS-MGFPGLQIG---HASQLLDT--QVPSPP         252
NR4A3   SLPLGAAAAAGSQAAALESHPYGLPLAKRAAPLAFPPLGLTPSPTASSLLGESPSLPSPP         281
NR4A1   QSPLKLFPSQATHQLG-EGESYSMPTAFPGLAPTSPHLEGS------GILDTPVTSTKAR         256
             *:    .:  .:      :  .   *   .     * *         :*.   ...


NR4A2   SRGSPSNEGLCAVCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRR         312
NR4A3   SRSSSSGEGTCAVCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRR         341
NR4A1   SGAPGGSEGRCAVCGDNASCQHYGVRTCEGCKGFFKRTVQKNAKYICLANKDCPVDKRRR         316
         *  .. ..** ********:***************************:*****:********


NR4A2   NRCQYCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKPKS-----PQEPSPPSPPVSLISA         367
NR4A3   NRCQYCRFQKCLSVGMVKEVVRTDSLKGRRGRLPSKPKSPLQQEPSQPSPPSPPICMMNA         401
NR4A1   NRCQFCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKPKQ-------PPDAFPANLLTS          367
         ****:*******:**********************.          .* : *  ::.:


NR4A2   LVRAHVDSNPAMTSLDYSRFQANPDYQMSGDDTQHIQQFYDLLTGSMEIIRGWAEKIPGF         427
NR4A3   LVRALTDSTP--RDLDYSRYCP-TDQAAAGTDAEHVQQFYNLLTASIDVSRSWAEKIPGF         458
NR4A1   LVRAHLDSGPSTAKLDYSKFQELVLPHFGKEDAGDVQQFYDLLSGSLEVIRKWAEKIPGF         427
         ****  ** *   .****::         .  *: .:****:**:.*:::  * ********
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Figure 6 Clustal sequence alignment of human NR4As. Shaded sequences correspond to the DNA binding domain where homology among NR4A1-3 is very high.

stronger activity over monomers[133]. The crystallographic analysis of NR4A receptors suggests that the members of this subfamily are constitutively activated: their LBD is almost completely occupied by bulky aminoacid side chains conferring a 3D structural conformation similar to that of agonist-bound receptors[132,133]. Consequently, unlike others NR, the activity of NR4As is not regulated by stimuli through a ligand binding but instead by modulation of their expression or *via* post-translational modifications[132,133]. Expression of these receptors is induced by a range of stimuli, including stimuli associated with metabolic functions, such as: fatty acid, growth factors, prostaglandines, membrane depolarization, cold, glucose, cholesterol, TZD and hormones[135]. Other hormones may regulate NR4A transcriptional activity interacting with the NR4 heterodimers, such 9-*cis*-RA on NR4A: RXR dimers, and new molecules have been identified acting as agonists or antagonists[96,97,136].

The involvement of NR4A2 in pancreatic cancer *in vitro* has been reported by two papers[133,136]. NR4A2 is highly expressed in many cancer cell lines including Panc1 and Panc28 pancreatic cancer cells. Structure-dependent activation of NR4A2 by a series of C-DIM analogs, especially a p-bromophenyl analog (DIM-C-pPhBr), alters the expression of NR4A2 target genes; among the altered genes the drug determines a NR4A2-dependent repression of neuropilin (NP)-2 in both cell lines, whereas it induces the expression of NP-1 in PANC28 but not PANC1[136]. NPs are important in the progression of pancreatic cancer and are currently seen as potential target for PDAC treatment[137] and it is conceivable that differential expression of these molecules by NR4A2 might be important for PDAC development. Moreover, in PC3 cells NR4A2 acts as a pro-

survival antiapoptotic factor[133]: silencing of the receptor greatly reduced the anchorage independent growth, with minimal effect in anchorage-dependent growth, largely due to increase anoikis; NR4A2 silencing also impaired the formation of tumors in nude mice[133].

More data link NR4A1 to pancreatic cancer, depicting a complex mechanism of action where Nur77 acts as having "two faces", being pro-survival and anti- and pro-apoptotic at the same time.

NR4A1 is expressed as a nuclear protein in pancreatic cancer cells[136,138-142] and was found to be overexpressed in PDAC tissues primarily in the nucleus, whereas 83% of non-tumor pancreatic tissues did not express it[142].

c-DIMs are a class of molecules that activate PPARγ and might act on NR4A receptors[136,138,139]; a c-DIM, specifically the 1,1-*Bis*(3'-indolyl)-1-(p-anisyl)methane (DIM-C-pPhOCH3), is the first identified Nur77 agonist[139]. DIM-C-pPhOCH3 activates GAL4-Nur77 chimeras expressing wild-type and the ligand binding domain of Nur77. In Panc-28 pancreatic cancer cells, Nur77 agonists decrease cell survival activating the cell death pathways, including tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and poly(ADP-ribose) polymerase (PARP) cleavage. Activation of TRAIL and PARP was further confirmed using Nur77 siRNA in Panc-28 cells. Nur77 agonists also inhibit tumor growth *in vivo* in athymic mice bearing Panc-28 cell xenografts[139]. DIM-C-pPhOCH3 arrests pancreatic cancer cell in G0-G1, by a Nur77-dependent, but KLF-4-independent, expression of cyclin-dependent kinase inhibitor p21. Interestingly, regulation of p21 does not require the presence of Nur77 response elements but involve a GC-rich promoter region and requires the presence of the Sp proteins Sp1

Figure 7 Nur77 acts as a tumor suppressor and as tumor promoting gene in pancreatic ductal carcinoma, inducing pro-apototic and anti-proliferative genes or repressing pro-survival genes. Dashed lines: Effects yet to be demonstrated in pancreatic ductal adenocarcinoma (PDAC). TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; PARP: Poly(ADP-ribose) polymerase; RXR: Retinoid X receptor; ER: Endoplasmic reticulum.

and Sp4, but not Sp3[140]. Microarray analysis of L3.6pL pancreatic cancer cells treated with DIM-C-pPhOCH3 demonstrated a NR4A1-dependent induction of genes associated with metabolism, homeostasis, signal transduction, transcription, stress, transport, immune responses, growth inhibition and apoptosis. Among the most highly induced growth inhibitory and proapoptotic genes were activating transcription factor 3 (ATF3), p21, cystathionase, dual specificity phosphatase 1 and growth differentiation factor 15. Furthermore, DIM-C-pPhOCH3 induced Fas ligand and TRAIL, the latter with a ATF3 dependent mechanism[141].

Although activation of Nur77 by specific c-DIM suggests that Nur77 is a tumor suppressor, experiments with the antagonist 1,1-*bis*(3'-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH) suggest the contrary[142]. Blocking of endogenous Nur77 results in increased cell death and reduced cell proliferation; moreover expression of anti-apoptotic genes Bcl-2 and Survivin is also reduced. When administered *in vivo*, DIM-C-pPhOH inhibits tumor growth, acting on the same antiapoptotic markers observed *in vitro*. Survivin is overexpressed in pancreatic cancer[143,144] and its expression increases during PanIN progression to PDAC[144]. Transcriptional regulation of Survivin by Nur77 is Sp1-dependent, paralleling the p21 regulation[140], and it is co-regulated by p300. Thus, activation of nuclear Nur77 by the agonist DIM-C-pPhOCH3 or inactivation by the antagonist DIM-C-pPhOH reduces proliferation and induces apoptosis through two different transcription pathways: the first involves the induction of expression of apoptosis promoter genes such as p21 and TRAIL, whereas the latter is dependent on suppression of pro-survival genes. Consequently, Nur77 acts both as a tumor suppressor and as a tumor promoting gene in pancreatic cancer (Figure 7).

Interestingly, Nur77 may act as an apoptotic inducer agent in several cancer cells after nuclear export[96,97,145-147]; although not yet described in PDAC it is conceivable that this extra-nuclear action may also be present in pancreatic cancer cells. Inducers of apoptosis, including 5'-fluorouracil which is used in PDAC treatment, stimulate nuclear export of Nur77 mediated by the export receptor CRM1[145,147]. NR4A1 nuclear export may also be induced by 9-*cis*-RA, requires RXRα as a carrier[97], and targets Nur77 to mitochondria[96]. Despite lacking classical mitochondria targeting sequences, Nur77 might translocate to mitochondria in response to cell death stimuli, through interaction with anti-apoptotic Bcl-2[147]. Bcl-2 acts forming channels in the mitochondria membrane to regulate apoptosis[148]. The interaction Bcl-2/Nur77 is mediated by the N-terminal loop of Bcl-2 and by the NR4A1 LBD: this binding induces a conformational change that exposes the BH3 region of Bcl-2, resulting in its transformation in an inducer of apoptosis[147]. Interestingly, Nur77 may also translocate to other organelles, specifically endoplasmic reticulum (ER). Cell treatment with CD437 induces a nucleus-cytoplasmic translocation of Nur77, followed by ER localization: this requires again the interaction with Bcl-2 and triggers the release of Ca^{2+} from ER inducing apoptosis. Again, this effect has been demonstrated in human neuroblastoma, esophageal squamous carcinoma and hepatocarcinoma cells[145,146] but not in PDAC cells (Figure 7).

LRH-1 in pancreatic cancer
LHR-1 (NR5A2) is an orphan NR that is essential during development and necessary in the adult for the function of the pancreas, liver, intestine, and ovary[149,150]. LHR-1 recognizes specific DNA sequences to whom it binds as monomere[151].
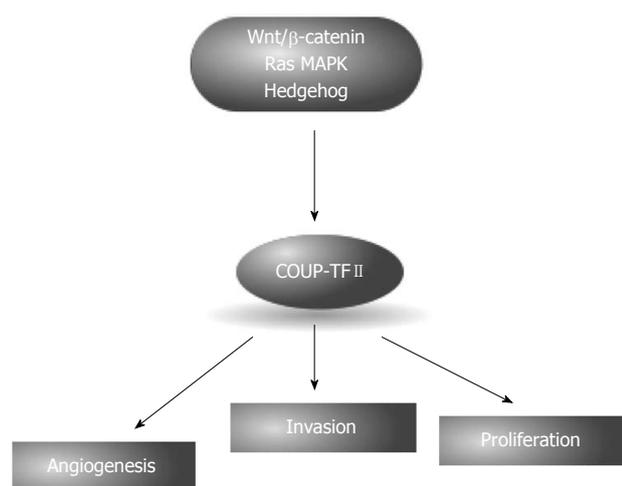
Figure 8 Chicken ovalbumin upstream promoter transcription factor Ⅱ is involved in the regulation of angiogenesis, invasion and tumor proliferation. Expression of chicken ovalbumin upstream promoter transcription factor Ⅱ (COUP-TFⅡ) is induced by several pathways altered in pancreatic ductal carcinoma, including Wnt/β-catenin, RAS-MAPK and Hedgehog.

The status of LHR-1 as "orphan" is debated and some scientists suggest that it may be classified as "adopted"[132,151]. The structure of the mouse LHR-1 LDB shows an active conformation with a large hydrophobic, but empty, ligand binding pocket resulting in a constitutive active receptor[132]; instead, the crystallographic analysis of the human LHR-1 revealed the presence of phosphatidyl inositol in the binding pocket[151]. Phosphatidyl inositol is required for activation[151] but it is not clear if it may enter and leave the pocket acting as a proper ligand[132]. Nonetheless, new molecules acting as antagonists have been recently identified by screening of commercially available compounds[152].

Chromatin immunoprecipitation-seq and RNA-seq analyses revealed that LRH-1 directly induces expression of genes encoding digestive enzymes and secretory and mitochondrial proteins and cooperates with the pancreas transcription factor 1-L complex in regulating exocrine pancreas-specific gene expression[153].

LHR-1 is important in maintaining acinar identity but is not required for acinar development[154] and mice with a selective deletion of LHR-1 in the pancreas did not display histological abnormalities[153-155]. A genome-wide association study conducted in pancreatic cancer patients and unaffected controls identified 5 SNP in the vicinity of NR5A2 associated with the risk of PDAC[156], that were confirmed by later studies[47,157,158].

In normal human pancreas, the LRH-1 protein is expressed at low levels in the nucleus and cytoplasm of both acini and ducts cells; in contrast PDAC show heightened levels of the protein and in some neoplastic cells the receptor appeared to localize predominantly in the cytoplasm[159]. An increased presence of LRH-1 was also detected in the acinar cells affected by pancreatitis and in PanIN lesions[159]. Overexpression of the receptor was also detected in pancreatic cancer cells *in vitro*[159].

Treatment of pancreatic cancer cells *in vitro* with LHR-1 antagonists or with LHR-1 siRNA significantly inhibits cell proliferation inducing a G0/G1 block associated with a reduction of of cyclins D1 and E1[152,159], suggesting that *in vitro* LHR-1 promotes tumor proliferation. *In vivo*, selective inactivation of one NR5A2 allele in pancreatic epithelial cells is sufficient to cause impaired recovery from pancreatitis[154] and conditional pancreatic deletion of LHR-1 leads to destabilization of the mature acinar differentiation state, increased inflammation, ADM and loss of regenerative capacity following acute caerulein pancreatitis[154,155]; loss of both alleles also dramatically accelerates the development of oncogenic Kras driven ADM and PanIN lesions[154,155].

The *in vivo* studies clearly show that LHR-1 inhibits the ductal transformation of adult acinar cells by mutant Kras and prevent PDAC progression; however *in vitro* studies show that NR5A2 promotes, instead of inhibiting, tumorigenesis. It has been hypothesized that NR5A2 exercises an inhibitory action in the early phase of PDAC development, blocking RAS with unknown mechanisms, while it will have an opposite effect later on[160].

COUP-TFⅡ expression predicts survival in PDAC
COUP-TFⅡ is a orphan nuclear receptor encoded by the NR2F2 gene localized in the chromosome region 15q26, a region frequently amplified in pancreatic cancer[14], and it is a down target of multiple pathways altered in pancreatic cancer[46,161-163]. In mouse two different transcription variants are described whereas in human at least four different variant are expressed. They differ in the N-terminal region and only one variant presents the structural features of NR being the others without the DBD. The role of these variants is not fully understood and two recent papers gave contradictory results describing one of the DBD lacking forms acting either as enhancer of COUP-TFⅡ transcriptional activity or as a repressor, increasing the cytoplasmic localization of full length COUP-TFⅡ, suggesting a cell specific function for this truncated NR[164,165]. COUP-TFⅡ exists in a autorepressed conformation that prevents recruitment of coactivators, and might respond to retinoids that promote COUP-TFⅡ to recruit coactivators[166]. Full length COUP-TFⅡ exerts an important role during development and in adulthood[167], and it is implicated in the progression of various type of cancers[168]. COUP-TFⅡ is expressed at low levels in adult normal exocrine pancreas[168,169] and recently we demonstrated its involvement in pancreatic cancer *in vitro* and *in vivo*[168] (Figure 8). COUP-TFⅡ was expressed in 69% of tested primary samples correlating with the presence of lymph and distant metastasis as well as clinical stage; PDAC patients stained positive for the NR showed a significant reduction of survival compared to NR-negative patients. *In vitro* silencing of COUP-TFⅡ reduces the cell growth and invasiveness and it strongly inhibits angiogenesis, an effect mediated by the regulation of VEGF-C. The reduced proliferation is associated with a block in G1 and

Table 2 Expression of nuclear receptors and clinical trials		
Nuclear receptor	**Expression in primary PDAC**	**Clinical trials and results**
PPAR (-α, -β, -γ)	PPARα: Unknown PPARβ/δ: overexpressed; expression correlates with tumor stage, recurrence, and distant metastasis PPARg: maybe overexpressed; expression correlates with shorter overall survival	None for PPARa and β/δ; PPARγ: TZD apparently reduce the risk of PDAC but PPARγ agonists do not improve survival
RAR and RXR (-α, -β, -γ)	Yes (with the exception of RARb that is apparently lost during cancer development)	13-*cis*-RA in combination with IFN-γ: prolonged stable disease as well no improvement have been reported
AR	Yes	Phase Ⅱ trials with the antagonist flutamide: increase in survival as well no effect have been reported
ER (-α, -β)	Yes	Tamoxifen with no benefit
NR4A1, NR4A2, NR4A3	NR4A1: overexpressed NR4A2 and NR4A3: unknown	None
LHR-1	Overexpressed	None
COUP-TF Ⅱ	Overexpressed; expression correlates with shorter overall survival, tumor stage, and presence of metastasis	None

PPAR: Peroxisome proliferator-activated receptor; PDAC: Pancreatic ductal carcinoma; TZD: Thiazolidinediones; IFN: Interferon; RAR: Retinoic acid receptor; RXR: Retinoid X receptor; AR: Androgen receptor; ER: Estrogen receptor; LHR-1: Liver receptor homologue-1 receptor; COUP-TF Ⅱ: Chicken ovalbumin upstream promoter transcription factor Ⅱ.

decreased expression of E2F1, but not apoptosis; moreover, COUP-TF Ⅱ silencing reduces OCT4 and increases Nanog expression. *In vitro* effects were confirmed in nude mice where COUP-TF Ⅱ silencing reduces tumor growth by 40%[168].

CONCLUSION

PDAC is a devastating disease originating from well defined genetic alterations. However, due to the its subtle nature, the lack of efficient diagnostic methods and of effective drugs it is a deadly disease with a dismal prognosis. NR are ligand-regulated transcription factors functionally involved in important cellular functions ranging from regulation of metabolism, to growth and development. Given the nature of their ligands, NR are very tempting drug targets and their pharmacological modulation has been widely exploited. There are now clear evidences that both classical ligand-activated and orphan NR are involved in the pathogenesis of pancreatic cancer disease from its very early stages. From the review of the literature PPARs, RARs, RXRs, AR, ERα and ERβ and the orphan NR Nur, COUP-TF Ⅱ and LHR-1 show striking connections with PDAC development, that for certainty will need more experimental confirmations, especially for the orphans. Although clinical application of NR modulators in the PDAC treatment still suffers from failure (Table 2), a more comprehensive analysis of NR action in PDAC could lead to the identification of novel therapies for this disease.

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Early detection and prevention of pancreatic cancer: Is it really possible today?

Marco Del Chiaro, Ralf Segersvärd, Matthias Lohr, Caroline Verbeke

Marco Del Chiaro, Ralf Segersvärd, Matthias Löhr, Division of Surgery, Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institute, 141 86 Stockholm, Sweden

Caroline Verbeke, Division of Pathology, Department of Laboratory Medicine, Karolinska Institute, 141 86 Stockholm, Sweden

Author contributions: Del Chiaro M and Verbeke C designed research and wrote the paper; Segersvärd R and Löhr M contributed to the research.

Correspondence to: Marco Del Chiaro, MD, PhD, Associate Professor of Surgery, Division of Surgery, Department of Clinical Science, Intervention and Technology (CLINTEC), Karolinska Institute, 141 86 Stockholm, Sweden. marco.del-chiaro@karolinska.se

Telephone: +46-85-8580000   Fax: +46-85-8586366

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Abstract

Pancreatic cancer is the 4th leading cause of cancer-related death in Western countries. Considering the low incidence of pancreatic cancer, population-based screening is not feasible. However, the existence of a group of individuals with an increased risk to develop pancreatic cancer has been well established. In particular, individuals suffering from a somatic or genetic condition associated with an increased relative risk of more than 5- to 10-fold seem to be suitable for enrollment in a surveillance program for prevention or early detection of pancreatic cancer. The aim of such a program is to reduce pancreatic cancer mortality through early or preemptive surgery. Considering the risk associated with pancreatic surgery, the concept of preemptive surgery cannot consist of a prophylactic removal of the pancreas in high-risk healthy individuals, but must instead aim at treating precancerous lesions such as intraductal papillary mucinous neoplasms or pancreatic intraepithelial neoplasms, or early cancer. Currently, results from clinical trials do not convincingly demonstrate the efficacy of this approach in terms of identification of precancerous lesions, nor do they define the outcome of the surgical treatment of these lesions. For this reason, surveillance programs for individuals at risk of pancreatic cancer are thus far generally limited to the setting of a clinical trial. However, the acquisition of a deeper understanding of this complex area, together with the increasing request for screening and treatment by individuals at risk, will usher pancreatologists into a new era of preemptive pancreatic surgery. Along with the growing demand to treat individuals with precancerous lesions, the need for low-risk investigation, low-morbidity operation and a minimally invasive approach becomes increasingly pressing. All of these considerations are reasons for preemptive pancreatic surgery programs to be undertaken in specialized centers only.

Core tip: Pancreatic cancer is the 4th leading cause of cancer-related death in Western countries. Considering the low incidence of pancreatic cancer, population-based screening is not feasible. This review analyzes the possibility to identify a population at risk for pancreatic cancer and the strategies for clinical screening and prevention.

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INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer-related death in Western countries. It ranks amongst the most lethal cancers and has a mortality rate that nearly equals the incidence rate and an overall 5-years survival of approximately 5%[1-3].

During the last decades there has been an overall reduction in cancer-related mortality in Western countries, in particular for lung, breast, colorectal and prostate cancer[1,4]. In contrast, mortality rates increased for PC, and the prediction for the year 2013 shows the same trend[4].

The success in reducing cancer mortality for some of the human solid tumors is not only related to the discovery of new therapeutic agents, but to a significant extent it has also been the result of the development of early detection and prevention programs. Born from this concept is a new surgical approach for patients at risk of cancer: preemptive surgery. Preemptive surgery can be defined as the prophylactic removal of an organ at high risk for malignant transformation or the resection of a precancerous lesion or an "early" malignant neoplasm in an individual with a predisposition to cancer[5]. Today, preemptive surgery is recognized as a useful approach for the management of various premalignant lesions or conditions and for the prevention of cancer in high-risk individuals. For patients with high-grade dysplasia in Barrett's esophagus, esophagectomy is a therapeutic option to prevent esophageal cancer from developing[6-10]. Total gastrectomy can be performed to prevent gastric cancer in the rare case of familial gastric cancer[11-13]. Bile duct cyst resection or "early" liver transplantation in primary sclerosing cholangitis is proposed to reduce the incidence of cholangiocarcinoma[14,15]. Total thyroidectomy may be recommended for individuals with multiple endocrine neoplasia type 2/familial medullary carcinoma[16]. Preemptive bilateral mastectomy and possibly bilateral oophorectomy in female carriers of *BRCA1* or *BRCA2* mutations reduce the risk of ovarian cancer and breast cancer by more than 90%[17,18]. Patients with hereditary non-polyposis colorectal cancer or familial adenomatous polyposis can be advised to undergo prophylactic total proctocolectomy to prevent colon cancer development[19]. Furthermore, a population-wide screening program to detect premalignant lesions or early cancer is already implemented for different tumor types such as colorectal, breast and lung cancer[20-24].

Historically, PC was not considered a disease suitable for a preventive or early detection program given the aggressive biology and rapid progression of the tumor and the presumed low prevalence of high-risk individuals. Today, newly acquired knowledge regarding the biology and natural history of PC has changed this view. Even if PC is biologically aggressive at the time of diagnosis, it follows a multistep carcinogenesis process, which is very similar to that of other cancers (*e.g.*, colon cancer) and consists essentially of a steady progression through increasing grades of dysplasia[25]. Since this process takes about a decade before the neoplastic lesion becomes an invasive cancer[26], there is a considerable window of opportunity to potentially detect the tumor at an early (pre-)invasive stage[27].

Today it has also been recognized that some of the neoplastic precursor lesions, such as intraductal papillary mucinous neoplasm (IPMN) or pancreatic intraepithelial neoplasia (PanIN), can be detected at an early stage using currently available imaging techniques[28,29].

Even if population-based screening for PC is not considered cost-effective given the relatively low incidence of the disease[25], specific screening programs for subgroups of high-risk individuals are currently evaluated by the scientific community regarding the detection of precursor lesions or early cancers and the impact on PC-related mortality[29]. In this paper we analyze the conditions that are associated with an increased risk of PC, the groups of individuals potentially suitable for screening programs, the target lesions for screening, and the potential treatment for these conditions.

GROUPS AT RISK FOR PC

A wide range of conditions are associated with an increased risk of PC. Overall, these can be divided in two distinct groups, *i.e.*, hereditary and non-hereditary conditions.

Traditional non-hereditary conditions associated with an increased risk of PC

These non-hereditary conditions are frequently associated with potentially correctable life-style factors and habits. Although they are well-established risk factors for PC, the associated increase in risk is too low to justify screening of the affected individuals. Smoking, obesity, alcohol abuse and exposure to toxic substances (Table 1) are potentially all suitable for primary prevention. Further non-genetic conditions associated with an increased risk of PC are diabetes type 1 and 2, chronic pancreatitis and a history of peptic ulcer (Table 1)[30-36]. Only the risk associated with chronic pancreatitis seems to be sufficiently high to justify screening of affected individuals.

Novel non-hereditary lesions and conditions associated with an increased risk of PC

The majority of patients within this group suffer from a pancreatic cystic neoplasm, in particular IPMN or mucinous cystic neoplasia (MCN), which bear an established risk of malignant transformation. Furthermore, recent data indicate that patients who underwent solid organ transplantation also have an increased risk of PC.

Pancreatic cystic neoplasms

Pancreatic cystic neoplasms (PCN) are common diseases with an estimated prevalence in the general population of approximately 20%[37,38]. A wide range of different cystic tumors has been described[39], but IPMN and MCN seem to be the most prone to undergo malignant transformation. Furthermore, the incidence of IPMN appears to be high in individuals with a familial risk for PC[40] and,

Table 1 Non-genetic factors associated with an increased risk to develop pancreatic cancer		
Risk factors	Estimated overall risk	Ref.
Smoking	1.75	[37,38]
Overweight	1.12 per increased 5 kg/m²	[39-41]
Alchohol abuse	1.2	[42,43]
Type 1 diabetes	2.0	[44]
New onset type 2 diabetes	2.0	[45]
Chronic pancreatitis	14.0	[46]
Exposure to nickel	1.9	[47]
Previous gastric ulcer	1.8	[48]

Table 2 Most important genetic syndromes associated with an increased risk of pancreatic cancer			
Syndrome	Gene	Relative risk	Risk at age of 70
Familial pancreatic cancer	Unknown		
1 or more first degree relative(s)		9	4%
1 first degree relative		4.5	2%
2 first degree relatives		6.4	3%
3 or more first degree relatives		32	16%
Peutz Jeghers syndrome	LKB1/STK11	132	30%-60%
Hereditary pancreatitis	PRSS1	50-70	40%
Familial atypical multiple mole melanoma	CDKN2A/p16	34-39	17%
Breast and ovarian cancer syndrome	BRCA1/BRCA2	2.3-10	1%-5%
Cystic fibrosis	CFTR	5.3	< 5%
Hereditary non-polyposis colon cancer	MSH2, MLH1, MSH6, PMS, PMS2	4.7	< 5%
Familial adenomatous polyposis	APC	4.5	2%

LKB1/STK1: Liver kinase B1/serine-threonine kinase 11; PRSS1: Protease serine 1; CDKN2A/p16: Cyclin-dependent kinase Inhibitor 2A; BRCA1/BRCA2: Breast cancer type 1 and 2 proteins; CFTR: Cystic fibrosis transmembrane conductance regulator; MSH2: DNA mismatch repair protein Msh2; MLH1: MutL homolog 1, colon cancer, nonpolyposis type 2; MSH6: MutS homolog 6; PMS, PMS2: Mismatch repair endonuclease.

conversely, a positive family history for PC is a risk factor for IPMN development[41]. The risk of cancer in IPMN patients ranges from 24% in IPMN involving the branch ducts to over 60% in lesions involving the main pancreatic duct[42]. For this reason, follow-up or preventive surgery of these neoplasms is recommended[39,42].

Transplanted patients
Individuals who underwent organ transplantation have recently been identified as being at risk of developing PC. Large cohort studies of transplanted patients in the United States have convincingly demonstrated that the immunosuppressive regimen used for solid organ transplantation is associated with a risk of PC, which significantly exceeds that of the general population[43]. Furthermore, following transplantation, patients seem to be at an increased risk to develop pancreatic neoplastic precursor lesions such as IPMN[44].

Hereditary conditions associated with an increased risk of PC
Three major groups of hereditary conditions can be considered associated with an increased risk of PC: a strong family history of PC, called familial pancreatic cancer (FPC), hereditary neoplastic and hereditary non-neoplastic syndromes in which PC is one of the phenotypic manifestations[45].

FPC
Even if a susceptibility gene for FPC has not been identified yet, the distribution of PC in some families meets the criteria for autosomal dominant transmission with reduced penetrance. In members of such kindreds, the risk to develop PC increases with the number of affected family members. The relative risk ranges from 4.5-fold in case of a single affected first-degree relative to 32-fold if 3 or more first-degree relatives are affected[46]. The definition of FPC is not well established yet, but an individual can be considered at risk if there are at least two first-degree relatives affected by PC, or if three or more relatives are affected regardless of the degree of relationship[47,48] (Table 2). According to some prospective observational studies based on screening programs[49], individuals at risk are found to have an increased incidence of neoplastic precursor lesions (PanIN and IPMN).

Breast and ovarian cancer syndrome
This syndrome, caused by mutations of the BRCA1 or BRCA2 genes, is associated with a 2.3- to 10-fold increased risk of PC[50,51] (Table 2). The risk to develop PC appears to be higher in individuals of Ashkenazi Jewish descent[52]. BRCA 2 mutations are also identified in about 13%-17% of the families diagnosed with FPC who do not meet the inclusion criteria of the breast and ovarian cancer syndrome[53,54].

Familial atypical multiple mole melanoma
Familial atypical multiple mole melanoma (FAMMM) is associated with a mutation of the *CDKN2A* gene. The syndrome is also associated with extra-cutaneous tumors, and PC is present in 25% of individuals who carry this mutation[55]. The relative risk to develop PC for individuals with FAMMM is 34- to 39-fold higher than in the general population[56,57] (Table 2). In a recent report from the German Familial Pancreatic Cancer surveillance program, patients with FAMMM were found more prone than patients with FPC to develop PC directly, *i.e.*, without the development of clinically detectable precursor lesions[58].

Peutz-Jeghers syndrome
Peutz-Jeghers syndrome (PJS) is an autosomal dominant hereditary disease that causes increased susceptibility for the development of various tumor entities. In particular, the risk of gastrointestinal tumors such as esophageal, small bowel, colorectal and pancreatic cancer is increased.
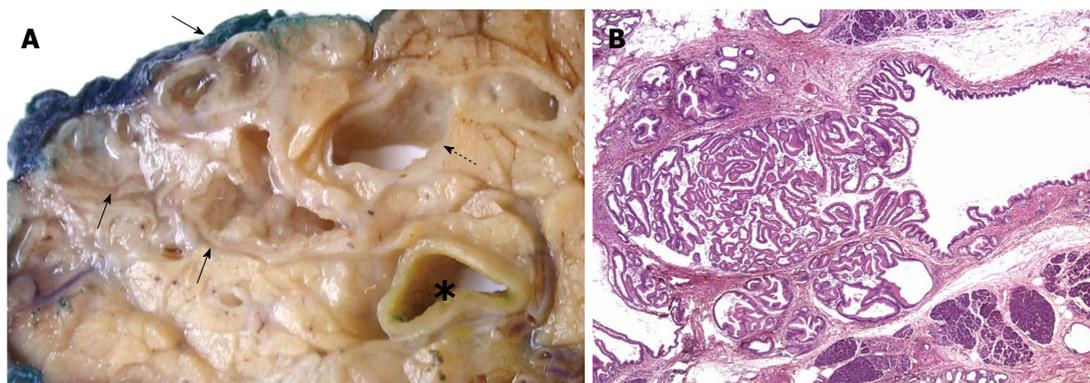
Figure 1 Intraductal papillary mucinous neoplasia. A: The main pancreatic duct (dotted arrow) and a cluster of branch ducts (arrows) are dilated, and particularly the latter contain mucus and a papillary proliferation on the duct walls (asterisk, common bile duct); B: Partial involvement of a pancreatic branch duct by the papillary proliferation of neoplastic epithelium characteristic of intraductal papillary mucinous neoplasia (HE, 20 × magnification).

Table 3 Characteristics related to epithelial subtype in intraductal papillary mucinous neoplasia

	Epithelial subtype			
	Gastric	**Intestinal**	**Pancreatobiliary**	**Oncocytic**
Location	Branch duct > main duct	Main duct > branch duct	Branch duct > main duct	Branch duct > main duct
Dysplasia	LGD/IGD	IGD/HGD	HGD	HGD
Present with invasive carcinoma	15%	30%-60%	60%-75%	25%
Type of invasive carcinoma	Conventional (tubular)	Colloid or conventional (tubular)	Conventional (tubular)	Oncocytic or conventional (tubular)

LGD: Low-grade dysplasia; IGD: Intermediate-grade dysplasia; HGD: High-grade dysplasia.

PJS is caused by a mutation of the STK11 gene[59]. The risk to develop PC is 132-fold higher in these patients compared to the general population[60] (Table 2).

Hereditary pancreatitis

Of the various types of hereditary pancreatitis (HP) described, only HP associated with a mutation of the *PRSS1* gene seems to bear a significantly increased risk of PC. Patients suffering from this condition usually present with early-onset chronic pancreatitis and a family history of chronic pancreatitis associated with PC. The relative risk to develop PC is 50-70[61,62] (Table 2).

Other hereditary conditions associated with an increased risk of PC

An up to 5-fold relative risk is associated with other hereditary syndromes. For hereditary non-polyposis colon cancer (HNPCC) or Lynch Syndrome II[63-65] the risk is approximately 4.7, for cystic fibrosis it is 5.3[66], 3 for ataxia telangiectasia[67] and 4.5 for familial adenomatous polyposis (FAP)[68,69]. Especially FAP patients are to be considered for preemptive pancreatic surgery, because premalignant lesions of the duodenum generally require the same surgical approach.

PRECURSOR LESIONS OF PANCREATIC CANCER

For several decades PanIN and IPMN have been established as precursor lesions of PC. However, it is only recently that their biology and significance are unfolding.

IPMN is, as the name indicates, a neoplastic proliferation within the pancreatic duct system that is characterized by a variable degree of papillary architecture and mucin production[70]. The tumour cell proliferation and mucin secretion cause duct dilatation, which is the major macroscopic and radiological feature of this tumour entity (Figure 1). Based on which part of the pancreatic duct system is involved, IPMNs are divided into main-duct, branch-duct or mixed-duct type. The neoplastic epithelium can be of gastric, intestinal, pancreatobiliary or oncocytic type[71]. Over time, IPMNs can develop increasingly dysplastic features (graded as low, intermediate and high) and eventually transform into invasive adenocarcinoma of tubular, colloid or the rare oncocytic type. Whereas the first cancer type is morphologically and prognostically identical to conventional PC, the latter two are more indolent. Interestingly, the various features of IPMN are interrelated, as outlined in Table 3. Recent studies indi-

PanIN-1 | PanIN-2 | PanIN-3
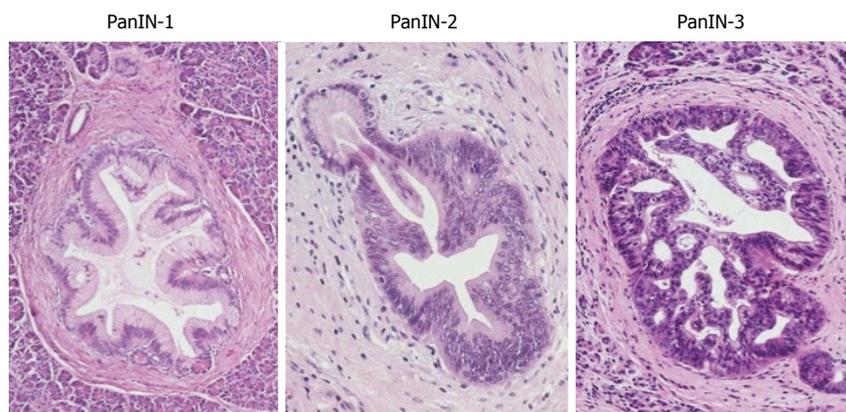
Figure 2 Pancreatic intraepithelial neoplasia. Small pancreatic branch ducts are involved by a low-papillary proliferation of neoplastic columnar epithelium showing mild, moderate and severe dysplasia corresponding to pancreatic intraepithelial neoplasia (PanIN)-1, PanIN-2 and PanIN-3.
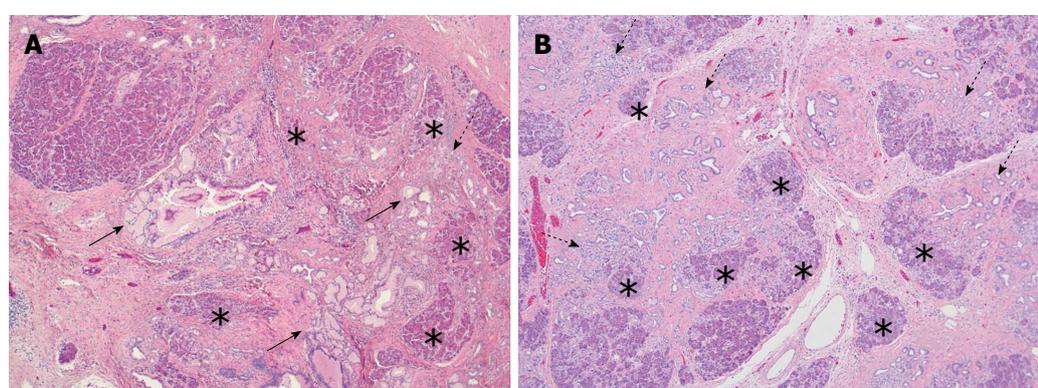
Figure 3 Lobulocentric atrophy. A: Lobules of acinar parenchyma are atrophic (asterisk) and partially replaced by tubular structures (so-called acinar to ductal metaplasia; dotted arrow) and fibrosis. Note the foci of PanIN-1 in the centre of the changes (arrows); B: Lobulocentric atrophy of neighbouring lobules (asterisk) results in a large area of fibrosis with tubular structures (dotted arrows) but without PanIN-lesion.

cate a more complex relationship between IPMN and invasive PC. The latter may not only develop through direct malignant transformation of the IPMN proper, but seems also to occur more frequently concomitant with (but topographically separate from) an IPMN, in particular branch-duct IPMN of gastric epithelial type[72-74].

PanIN is also characterized by a neoplastic intraductal proliferation, but in contrast to IPMN, the neoplastic epithelium is flat to low-papillary, and mucin secretion is not a prominent feature[70]. The epithelial type is mainly gastric, although intestinal, oncocytic and other variants can occasionally occur[72]. Three grades of dysplasia (PanIN-1, PanIN-2, PanIN-3) are usually distinguished (Figure 2). The lower grades (PanIN-1, -2) are a common finding in otherwise healthy pancreas after the age of 40[75,76] or in chronic pancreatitis[77-79]. In contrast, PanIN-3 is rare in the normal pancreas or chronic pancreatitis[76,80], but appears most commonly in pancreas with invasive ductal adenocarcinoma[81]. Both PanIN and IPMN are more common and more often multifocal in individuals with a strong family history of PC than in patients with sporadic disease, and the precursor lesions are of a higher grade in the former group[51,82,83].

While a certain morphological overlap exists between branch-duct IPMN of gastric type and PanIN[84,85], the main difference between PanIN and IPMN is the fact that the latter represents a macroscopically identifiable lesion[86], while PanINs are too small to be visualized by naked-eye inspection or imaging. However, it has been recently suggested that PanIN is associated by parenchymal changes[51,82,87], which may be detected by EUS[88,89]. These parenchymal changes are characterized by a combination of acinar cell loss, proliferation of small ductular structures and fibrosis, and have been coined as "lobulocentric atrophy" (LCA) (Figure 3A). The initial enthusiasm about the possibility to identify PanIN by means of detecting LCA has been dampened by recent novel developments regarding the causal relationship between both lesions. While it was first assumed that LCA is caused by the duct-obstructive effect of PanIN lesions[51,82,87], and the association between both seemed more or less obligatory, recent morphological and molecular evidence indicates that PanIN is one of the possible outcomes of LCA or the process that is often associated with LCA, so-called acinar-to-ductal metaplasia[90-92]. In the light of these recent discoveries, the use of LCA as a target for pancreatic screening of high-risk individuals requires more circumspect consideration. First, because PanIN is not the cause

Table 4 Incidence of cancer during follow-up of branch-duct intraductal papillary mucinous neoplasms of the pancreas			
Ref.	Year	Patients (*n*)	Incidence of cancer on IPMN
Lafemina *et al*[97]	2013	170	11.0%
Sahora *et al*[98]	2013	240	4.0%[1]
Khannoussi *et al*[99]	2012	53	3.7%
Arlix *et al*[100]	2012	49	0
Maguchi *et al*[101]	2011	349	2.6%[2]
Salvia *et al*[102]	2009	121	0
Tanno *et al*[103]	2008	82	1.2%

[1]Other 3.7% of patients developed a concurrent pancreatic cancer (PC); [2]Other 0.8% of patients developed a concurrent PC. IPMN: Intraductal papillary mucinous neoplasms.

Table 5 Summary of diagnostic yield of familial pancreatic cancer screening				
Ref.	Year	Syndrome(s)	Patients (*n*)	Yield
Brentnall *et al*[106]	1999	FPC	14	50%
Kimmey *et al*[107]	2002	FPC	46	26%
Poley *et al*[108]	2009	FPC, PJS, BRCA, p16, p53, HP	44	23%
Langer *et al*[109]	2009	FPC, BRCA	76	1.3%
Verna *et al*[110]	2010	FPC, BRCA, p16	51	12%
Ludwig *et al*[111]	2011	FPC, BRCA	109	8.3%
Vasen *et al*[112]	2011	P16	79	18%
Al-Sukhni *et al*[113]	2011	FPC, BRCA, PJS, p16, HP	262	7.3%
Schneider *et al*[114]	2011	FPC, BRCA, PALB2	72	15%
Canto *et al*[115]	2012	FPC, BRCA, PJS	216	43%

HP: Hereditary pancreatitis; FPC: Familial pancreatic cancer; PJS: Peutz-Jeghers syndrome; BRCA: Breast cancer.

of LCA, the association between both lesions is not 100%[93]. LCA is in fact a common finding in the ageing pancreas or in the context of various conditions[87,90] and may be present with or without associated PanIN (Figure 3B). Conversely, PanIN may well occur in the absence of LCA, *i.e.* remain undetectable on EUS examination. Second, there is so far no indication that the presence of LCA correlates with the grade of PanIN. In other words, EUS detection of LCA would still not provide sufficient information for patient management, as high-grade PanIN may be an indication for preventive surgery, whereas low-grade PanIN is not. Third, the accuracy with which fine needle aspiration (FNA) would be able to assess the PanIN-lesion presumed to be associated with the focus of LCA identified on EUS has not been evaluated. While a focus of LCA may be of varying size depending on whether a single pancreatic lobule or several neighbouring lobules are affected, the associated PanIN-lesion(s) may be present only focally and could thus be missed by EUS-guided FNA. From these considerations, it appears that sampling bias may represent a limitation to the successful identification of PanIN-lesions when screening individuals with an increased risk of PC.

SCREENING MODALITIES FOR INDIVIDUALS AT RISK

While various modalities are available to screen patients at risk of PC, it is currently not well defined who should be screened and how this could be done.

Individuals with a "non-hereditary risk" of PC
Among individuals with a "non-hereditary" risk of PC, patients suffering from chronic pancreatitis or PCN are currently enrolled for clinical screening.

Patients with chronic pancreatitis are usually entered into a screening program to follow the evolution of the disease and detect PC at an early stage[93]. Recently, a specific algorithm based on patient history and laboratory tests has been developed to identify those chronic pancreatitis patients that have developed early PC[94]. Traditionally, screening of these patients has been based

on imaging by MRI and CT scan. The use of EUS, alone or in combination with MRI, seems to offer a high accuracy in this particular patient group. The role of FNA during EUS is not conclusively defined[95], and diffusion-weighted MRI does not seem to facilitate the distinction between PC and chronic pancreatitis[96]. In the group of patients with PCN, MRI, CT scan and EUS, alone or in combination, are the most effective screening modalities. However, in view of the possible need for a prolonged screening program, CT scan is not recommended due to the risks associated with radiation exposure[39,42]. In particular for branch-duct IPMN (BD-IPMN), the surveillance strategy seems to be effective, as evidenced by the average detection rate of cancerization during follow-up, which lies between 0% and 11%[97-103] (Table 4).

Individuals with a "hereditary risk" of PC
Over the years, various surveillance programs have been developed for individuals with a "hereditary risk" of PC. Recently, MRI and EUS have become the most commonly used investigational modalities, whereas in the past CT scan and ERCP have also been used in this field[29]. Within this particular group, individuals affected by FAMMM deserve special mentioning. Indeed, in these individuals the development of PC follows a different pathway that is not preceded by PanIN and IPMN lesions[58]. Therefore, EUS should probably be preferred to, or used in combination with, MRI. Other methods of investigation that are potentially useful for the diagnosis of IPMN include pancreatoscopy and confocal laser microscopy. However, these methods are still experimental and cannot be used routinely for individuals with a hereditary risk of PC[104,105].

At present, the results of screening programs for PC are inconclusive. Most of the prospective studies performed so far report a highly variable detection rate of pancreatic findings, the yield ranging from 1% to 50% (Table 5)[106-115]. The significant divergence in detection rate is not only due to the use of different screening modalities but results also from differences in the defi-

nition of the concept "yield". Some studies declared the identification of "early cancer" (T1N0M0) or high-grade dysplastic precursor lesions as the goal of screening, whereas others also included IPMN with low- or intermediate-grade dysplasia or PanIN of any grade of dysplasia. Some of the surveillance protocols attempted at detecting PanIN lesions by EUS, based on their association with lobulocentric atrophy. However, this histological change in the pancreas is not specific for PanIN, the ability to recognize it is very operator-dependent, and the progression and natural history of this type of lesion is not well known in individuals at risk of PC[29,116]. Consensus has not yet been reached regarding the timing and inclusion criteria for a surveillance program. National and international guidelines suggest that every individual with a 5- to 10-fold relative risk should be considered for surveillance[28,29]. A further point of dissensus is the age at which an individual should be enrolled for screening. For patients at risk of FPC, age 40 or 50 has been proposed for the commencement of screening. However, an earlier age has been suggested for individuals at risk who smoke[29]. For patients with HP (PRSS1 mutation carriers), starting surveillance at the age of 40 has been recommended, considering the younger age of onset of PC in this particular patient group[29]. While no recommendations have been made regarding the age at which an individual could be discharged from a screening program, it seems appropriate that this should be determined by the individual's fitness for surgery. The exact timing of the screening procedures also lacks clear definition. In general, a yearly control is performed in patients without any finding on previous investigations. In case changes were detected that do not represent an indication for surgery, follow-up at 3- to 6-monthly intervals is generally recommended[29].

PRIMARY PREVENTION FOR INDIVIDUALS AT RISK OF PC

Unfortunately, primary prevention for individuals at risk of PC is currently not available. Removal of the pancreas based exclusively on a statistical risk is not recommended[29]. In some individuals, advice regarding a healthier life style can be given, for example cessation of smoking, a diet rich in fruits and vegetables, regular exercise, and weight reduction or, if indicated, increased vitamin D intake (> 600 IU)[117].

SURGERY FOR INDIVIDUALS AT RISK

Individuals with a "non-hereditary risk" of PC
Chronic pancreatitis: In patients with sporadic chronic pancreatitis, surgery is a second line option for the treatment of local complications and symptoms, if a conservative approach has failed[118]. Even if a number of different surgical procedures for chronic pancreatitis have been proposed in the literature[119,120], a radical pancreatic resection should be performed whenever a suspicion of

malignancy arises[121]. However, in selected cases, as for example of HP, some authors suggest early removal of the gland (total pancreatectomy) combined with auto-islet transplantation[122]. The rational of this approach, which cannot be considered the gold standard, is to treat the symptoms (mostly pain), eliminate the risk of cancer, and prevent the development of diabetes following total pancreatectomy. Today, this approach is also possible with a minimally invasive technique[123].

Pancreatic cystic neoplasms: The indications for surgery in patients with IPMN or MCN are spelled out in European and international guidelines[39,42]. For main-duct and mixed-type IPMN, surgical resection is always indicated because of the increased cancer risk. The extent of pancreatic resection should be planned based on the findings on preoperative imaging. In case of dilatation of the entire main pancreatic duct without signs of malignancy in the tail region, a pancreatoduodenectomy with frozen section of the pancreatic margin is recommended. Extension of the resection is indicated if intraoperative examination shows high-grade dysplasia. Low-grade dysplasia is not considered an indication, whereas intermediate-grade dysplasia represents a grey area in which extended resection is not strongly recommended and a judicious decision depends mainly on clinical considerations specific for the individual patient. Importantly, however, extended resection for high-grade dysplasia at the margin is not strongly recommended, if invasive carcinoma is present in the pancreatic head, the reason being that the cancer will determine the patient's outcome. Therefore, intraoperative frozen section examination may be useful if there is radiological suspicion of malignancy. Total pancreatectomy should not be considered based only of the extent of the duct dilatation, because the latter can be related to mere duct obstruction[39]. Regarding the indication for resection of BD-IPMN, recent guidelines[39] recommend a surgical approach in patients with signs or symptoms of malignancy (dilatation of the main pancreatic duct up to 6 mm, mural nodules, rapid increase in size, elevated levels of CA19.9) or a lesion measuring up to 4 cm in maximum diameter[39]. In case of multifocal disease, only the lesion with these particular features should be resected (partial pancreatectomy). A radical resection should be performed when malignant transformation is suspected. An algorithm that combines the current European and international guidelines is proposed in Figure 4. When signs of malignancy are not present but the diagnosis is not entirely clear or the patient presents with certain clinical risk factors, parenchyma-sparing resection has been suggested by some[124].

Transplanted patients with premalignant lesions: Even though specific guidelines regarding screening for pancreatic disease do not exist for transplanted patients, the increased risk of PC[43] and premalignant pancreatic lesions[44] seems to justify focused attention to this group of patients. Published in the literature is only a single
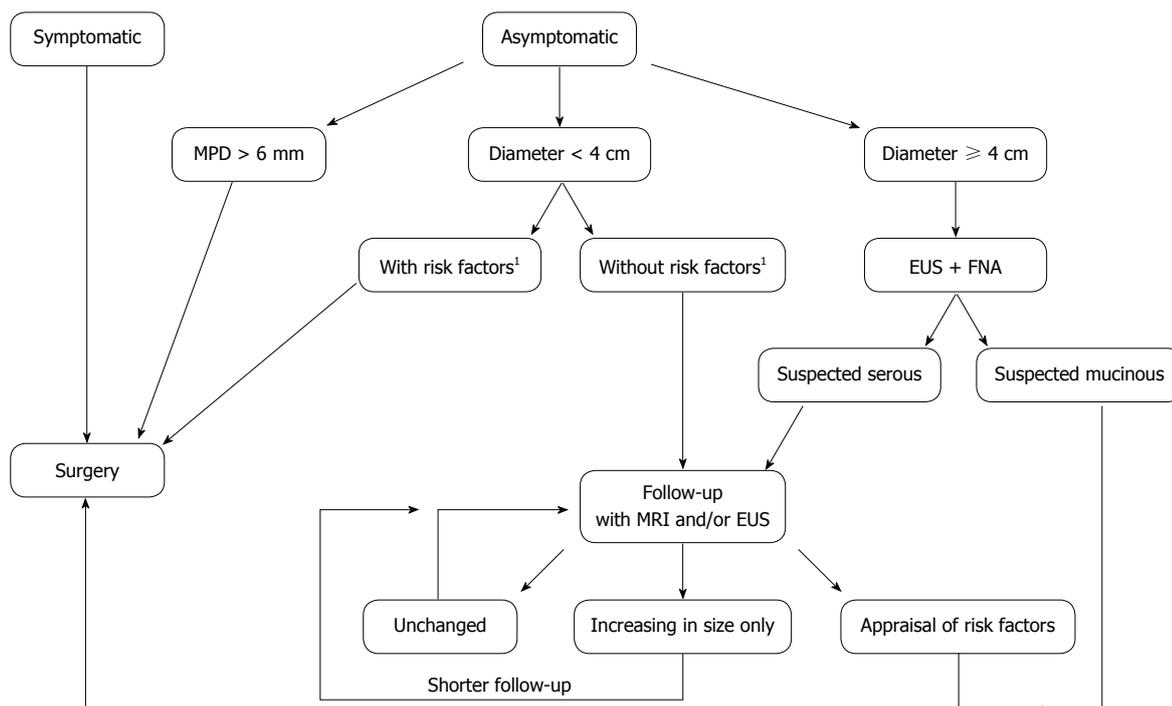
Figure 4 Algorithm that combines the current European and international guidelines for intraductal papillary mucinous neoplasia of the pancreas. FNA: Fine needle aspiration; MPD: Main pancreatic duct; EUS: Endoscopic ultrasound. Risk factors[1]: Mural nodules, increased serum levels of Ca 19.9, rapid increase in size.

study that analyzed the clinical history of premalignant lesions in transplanted patients and found no difference in terms of progression time compared to non-transplanted patients[125]. However, considering the short patient follow-up in this study and the natural history of the lesions under scrutiny, the data seem insufficient as a basis for the development of a strategy. A surveillance protocol for transplanted patients and the option of early parenchyma-sparing resection have been recently suggested[126].

Individuals with a "hereditary risk" of PC

At present, a surgical strategy for patients with FPC or other hereditary syndromes has not been well defined. In contrast to previous practice, when aggressive approaches such as total pancreatectomy and pancreas transplantation were proposed for patients with a positive family history and findings "suggestive" of dysplasia[127], a more conservative philosophy currently prevails. Even when national and international guidelines recognize PanIN lesions as potential targets for screening[29], they also underline the difficulty to obtain a correct diagnosis for these lesions and therefore the unlikely suitability of PanINs as targets for a clinical surveillance program.

The surgical results from international studies vary considerably (Table 6). The reasons for this discrepancy are differences in inclusion criteria, screening modalities and, most importantly, differences in indications for surgery[88,106-115].

Current international guidelines recommend surgery for patients at risk with defined solid lesions, cystic tumors that meet criteria for resection even in a population that is not high-risk, and histologically proven PanIN-3 lesions[29]. In high-risk individuals, the indication for surgery for cystic lesions can be adjusted according to the family history, the age and the patient's perception of the problem, as suggested by the European guidelines for cystic tumors of the pancreas[39]. The surgical treatment of patients at risk of PC should be undertaken in high-volume centers with specialization in this field[29].

DISCUSSION

The group of hereditary and non-hereditary conditions that are associated with an increased risk for PC has been well defined[30-36,42,43,46,50,51,56,57,60,62]. Even though there is general agreement that individuals with a relative risk of over 5-10 times that of the general population should be considered for enrollment in a clinical surveillance program[29], consensus regarding the latter is currently lacking. As a result, data from clinical trials in this particular area are conflicting[29]. In addition, a more fundamental reason lies at the root of divergent observations and results, namely the lack of knowledge about possible differences in natural history of the premalignant lesions that develop in the various hereditary and non-hereditary conditions of individuals at risk[28,29,42]. However, in everyday clinical practice, the relentless stream of patients with premalignant lesions of the pancreas or individuals with a genetic risk of PC who seek medical advice, represents a significant clinical burden. On one hand, there is a clear need from the pancreatologist's point of view to offer

Table 6 Surgical procedure performed in patients at "hereditary risk" for pancreatic cancer (*n*)

Ref.	Year	Resected	PanIN1	PanIN2	PanIN3	Pancreas cancer	Other bening lesions	Other malignant lesions	Benign IPMN	Malignant or high-grade displasia IPMN	Malignant lesions or high-grade dysplasia[1]
Brentnall *et al*[106]	2006	7	-	2	2	-		-	2	1	42.8%
Poley *et al*[108]	2009	3	-	-	-	3		-	-	-	100%
Verna *et al*[110]	2010	5	-	-	-	1	-		4	-	20%
Ludwig *et al*[111]	2011	6	1	1	1	1	-	-	2	-	16.7%
Vasen *et al*[112]	2011	7	-	-	-	7	-	-	-	-	100%
Al-Sukhni *et al*[113]	2011	4	-	-	-	1	-	1	2	-	50%
Schneider *et al*[114]	2011	9	1	1	1	1	3	-	2	-	22.2%
Canto *et al*[115]	2012	5	-	-	1	-	-	-	3	1	40%
Total		46	2	4	5	14	3	1	15	2	47.8%

[1]The percentage refers to all resected patients, not to all patients included in the study. IPMN: Intraductal papillary mucinous neoplasm; PanIN: Pancreatic intraepithelial neoplasia.

concrete advice, while on the other hand there is the pressing need to increase our knowledge about the natural history and biology of the lesions under scrutiny. For some of the conditions associated with an increased risk of PC, guidelines and protocols that provide the possibility to standardize treatment and give advice to patients, have already been established (*e.g.*, for cystic tumors or chronic pancreatitis)[41,44]. New discoveries for example regarding cystic tumors of the pancreas are very promising as they may allow prediction of the future evolution of the cystic tumors and thus enable the clinician to decide for surgical or non-surgical treatment[125]. For patients with a hereditary syndrome, the situation is more complex. In several countries, a surveillance program can be rolled out only in the context of a clinical trial, because the cost-effectiveness of surveillance programs for this particular group of high-risk individuals has not been demonstrated yet. Furthermore, only very limited data are available regarding the screening of other groups of patients at risk, such as transplanted patients[128]. Despite the apparently long road that still lies ahead, the recent progress that has been achieved regarding the understanding and management of premalignant lesions of the pancreas, and the role that pre-emptive surgery has acquired in cancer syndromes other than those affecting the pancreas, render a similar development for pancreatic tumors more than likely. Along with the steadily increasing number of patients that will be treated for premalignant lesions, a growing demand for technical perfection and minimally invasive approaches appears unavoidable[129]. At the same time, due to increasing patients' expectations, the need for a more evidence-based approach, and stricter cost-effectiveness regimes, the pancreatic team will be under increasing pressure to minimize diagnostic error and surgical risk and to optimize the use of limited resources in the health care system. For this reason, surveillance and treatment of individuals at increased risk of PC should be limited to high-volume and specialized centers with a specific clinical and research interest in preemptive pancreatic surgery.

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2014 ADVANCES IN PANCREATIC CANCER

Biomarkers for pancreatic cancer: Recent achievements in proteomics and genomics through classical and multivariate statistical methods

Emilio Marengo, Elisa Robotti

Emilio Marengo, Elisa Robotti, Department of Sciences and Technological Innovation, University of Piemonte Orientale, 15121 Alessandria, Italy
Author contributions: All authors contributed equally to the drafting of the manuscript.
Correspondence to: Emilio Marengo, Professor, Department of Sciences and Technological Innovation, University of Piemonte Orientale, Viale Michel 11, 15121 Alessandria, Italy. marengoe@tin.it
Telephone: +39-0131-360259 Fax: +39-0131-360250
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Abstract

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases. A valid alternative to the usual invasive diagnostic tools would certainly be the determination of biomarkers in peripheral fluids to provide less invasive tools for early diagnosis. Nowadays, biomarkers are generally investigated mainly in peripheral blood and tissues through high-throughput omics techniques comparing control *vs* pathological samples. The results can be evaluated by two main strategies: (1) classical methods in which the identification of significant biomarkers is accomplished by monovariate statistical tests where each biomarker is considered as independent from the others; and (2) multivariate methods, taking into consideration the correlations existing among the biomarkers themselves. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and prognostic performances which are superior to single markers in terms of sensitivity, specificity and robustness. Multivariate techniques are usually applied with variable selection procedures to provide a restricted set of biomarkers with the best predictive ability; however, standard selection methods are usually aimed at the identification of the smallest set of variables with the best predictive ability and exhaustivity is usually neglected. The exhaustive search for biomarkers is instead an important alternative to standard variable selection since it can provide information about the etiology of the pathology by producing a comprehensive set of markers. In this review, the most recent applications of the omics techniques (proteomics, genomics and metabolomics) to the identification of exploratory biomarkers for PC will be presented with particular regard to the statistical methods adopted for their identification. The basic theory related to classical and multivariate methods for identification of biomarkers is presented and then, the most recent applications in this field are discussed.

Core tip: Biomarkers are statistically identified as significant by: (1) classical statistical tests where each biomarker is independent from the others; and (2) multivariate methods that take into consideration the correlation among the biomarkers. This last approach provides pools of biomarkers with superior diagnostic and prognostic performances. Multivariate techniques are often applied with variable selection procedures to provide the smallest set of biomarkers with the best predictive ability. The exhaustive identification is instead a valid alternative since it can provide comprehensive information about the etiology of the pathology. The most recent applications of the omics approaches to the identification of biomarkers for PC are presented, with particular regard to the statistical methods adopted.

INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive and lethal neoplastic diseases; its early detection is therefore fundamental since surgery at an early disease stage is the preferred and most promising therapy. About 20% of patients can be operated on at time of diagnosis; the 5-year survival rate for not-operable patients is about 1%, while the 5-year survival after surgery is about 20% without an adjuvant therapy and about 25%-30% with the therapy[1-3]. The lack of early symptoms and the high aggressiveness are the main causes of late diagnosis and high mortality of this disease. Therefore, the search for biomarkers of early diagnosis is highly recommended to improve the early diagnostic rate, thus improving patients' prognosis.

Diagnosis is usually based on invasive techniques [ultrasound endoscopy (EUS), explorative laparoscopy or laparotomy] or on methods that can be at least inconvenient for patients [computed tomography (CT), magnetic resonance imaging (MRI), endoscopic retrograde or magnetic resonance cholangiopancreatography (ERCP and MRCP)][2].

A valid alternative would certainly be the determination of specific biomarkers in peripheral fluids in order to provide less invasive tools for early diagnosis. In this direction, the most recent efforts in the field of biomarker identification for PC are directed. A wide range of serum markers for PC has been reported[2,4] but few of them are exploited in clinical routine since they show low sensitivity and/or specificity in general. Bünger *et al*[2] reviewed about 43 serum biomarkers for PC divided into four main groups: carbohydrates (CA19-9, CA 50, CA 125, CA195, CA 72-4), carcinoembryonic antigens, other markers and the combination of different markers.

Together with diagnostic markers, great efforts have recently been made to identify predictive and prognostic biomarkers for PC. Tissue biomarkers for the prognosis of pancreatic ductal adenocarcinoma (PDAC) have recently been reviewed by Jamieson *et al*[4]. The considerations that drive the search for diagnostic biomarkers also apply for predictive and prognostic ones and the identification of markers from peripheral blood should be the best alternative to provide prognostic methods that are less invasive for patients. Nowadays, both diagnostic and prognostic/predictive biomarkers are generally investigated mainly in peripheral blood or tissues through high-throughput omics techniques. The results can be evaluated by two different strategies, namely by: (1) classical statistical methods consisting of the use/identification of significant biomarkers by monovariate statistical tests

where each biomarker is considered as independent from the others; and (2) multivariate methods, able to take into consideration the multivariate structure of the data and the correlations among the potential biomarkers. This last approach is very powerful since it allows the identification of pools of biomarkers with diagnostic and/or prognostic performance superior to single markers in terms of sensitivity, specificity and robustness.

It is important to point out that biostatistical methods can usually be defined as multivariate (several endpoints and several predictors) or multivariable (one endpoint, several predictors). In this specific context, the authors will generally apply the term multivariate to identify methods that allow the evaluation of the correlations between the variables, *i.e.*, their synergisms and/or antagonisms.

Multivariate techniques are usually applied with variable selection procedures[5,6] to provide a set of candidate biomarkers with the best predictive ability; however, standard selection tools are aimed at the identification of the smallest set of variables with the best predictive ability. It is the authors' opinion that exhaustivity should also be addressed[7]. Biomarkers are useful not only for diagnostic/prognostic purposes but also to better understand the etiology of pancreatic cancer. From this point of view, the exploitation of high-throughput methods provides a lot of information that should not be neglected. The exhaustive identification of all possible biomarkers showing large correlations could provide information about the overall mechanism of action of the disease, thus opening the way towards new therapeutic strategies.

In this review, the most recent applications of the omics approaches (proteomics, genomics and metabolomics) for the identification of biomarkers for pancreatic cancer will be presented, with particular regard for the statistical methods adopted for their identification, focusing especially on exploratory biomarkers. High-throughput techniques will probably be the future in the field of searching for exploratory biomarkers due to the great amount of information they convey. Moreover, the possibility of combining the results emerging from proteomic, genomic and metabolomic studies with clinical information can provide exhaustive panels of markers, thus improving their predictive performance with better sensitivity and specificity.

First, the theory of the classical and multivariate methods for identification of biomarkers will be presented, followed by the most recent applications in this field.

STATISTICAL METHODS

The statistical methods presented here can be divided into four main groups: (1) classical methods for identification of biomarkers based on monovariate approaches; (2) tools for biomarkers search based on multivariate approaches; (3) methods for the analysis of survival outcomes; and (4) other methods. Only the tools recently applied to the specific case of pancreatic cancer will be

Table 1 Statistical methods adopted in the identification of biomarkers for pancreatic cancer

Type of statistical method	Method adopted
Classical mono- and multi-variate methods	Student *t*-test (parametric)
	Mann-Whitney *U*-test (non-parametric)
	T² Hotelling
	ANOVA and MANOVA
	Bayes factors
Unsupervised pattern recognition methods	Principal Component Analysis
	Cluster Analysis
	Multidimensional Scaling
Supervised classification methods	SIMCA
	Ranking-PCA
	O-PLS
	CART
	Random Forests
Methods for determining survival outcomes	Kaplan Meyer functions
	Cox Regression
Other methods	PAM
	Metropolis algorithm and Monte Carlo simulation

PCA: Principal component analysis; SIMCA: Soft independent model of class analogy; PLS: Partial least squares; CART: Classification and regression tree.

briefly presented as an exhaustive treatment of all multivariate procedures in the field of searching for biomarkers is out of the scope of the present review.

CLASSICAL MONOVARIATE METHODS FOR IDENTIFICATION OF BIOMARKERS

The classical approach to the identification of markers of a specific disease is the evaluation of which variables show a different behavior between two groups of samples (control *vs* pathological, control *vs* drug-treated, *etc*.). The easiest statistical way to solve this problem is the application of classical statistical tests to each biomarker candidate separately and the calculation of the type I error that can be accomplished comparisonwise (for each hypothesis independently) or experimentwise (testing all hypotheses together). The second alternative is preferred since the type I error probability increases as the number of tests increases. The identification of significant markers is therefore accomplished by the Student's *t*-test for each variable independently and by applying a correction taking into account the number of multiple tests available: Bonferroni's method with subsequent modifications[8] or the corrections proposed by Dunn and Sikak and by Dunnet[9-11]. This approach is incorrectly defined as multivariate since it does not take into consideration the correlations eventually existing between the variables. The same approach can also be applied to non-parametric tests to be exploited when the number of samples is too small or when the assumptions at the basis of parametric tests are not verified. Among them, the most widespread in the biomedical field is the Mann-Whitney *U*-test[8].

An alternative is the exploitation of global tests aimed at demonstrating a global hypothesis (*e.g.*, the effect of a

therapy) considering all the tests simultaneously; an example is the Hotelling's T^2 test[8].

Classical procedures also comprise the approach based on the analysis of variance both in its two-way (ANOVA) or multi-way (MANOVA) versions[8]. In this case, it is also possible to compare more than two groups of samples.

An alternative to classical hypothesis testing is the use of the Bayes factors[12], providing a more robust approach. For comparing two hypotheses H₁ and H₂, this factor may be approximated as the ratio of the marginal likelihood of the data under the two hypotheses and can be interpreted as follows: B $\leqslant$ 0.1: strongly against H₁; 0.1 < B $\leqslant$ 1: against H₁; 1 $\leqslant$ B < 3: barely worth mentioning for H₁; 3 $\leqslant$ B << 10: substantially for H₁; B > 10: strongly for H₁.

MULTIVARIATE METHODS FOR IDENTIFICATION OF BIOMARKERS

Methods that can be properly defined multivariate are based on the comparison of two or more groups of samples taking into account the relationships between the variables, rather than considering them as independent. This approach is certainly more effective since it is fundamental to consider the synergic or antagonistic effects of different factors, *i.e.*, their interactions. Moreover, when independent tests are performed on several factors and a high correlation exists between them, the outcome of the test can be completely wrong[13]. The methods here presented belong to two approaches: (1) unsupervised methods (pattern recognition methods), in which no a priori information is assumed and the evaluation of the existence of groups of samples is suggested by the statistical method itself; and (2) supervised methods (classification tools), in which the a priori information is provided in terms of membership of each sample to a specific class: the statistical method is therefore aimed at the identification of the variables responsible for the separation of the samples in the different classes.

Here, only the methods most recently applied in the literature to the case of pancreatic cancer will be briefly discussed. The methods presented are listed in Table 1.

UNSUPERVISED AND PATTERN RECOGNITION METHODS

Principal component analysis

Principal component analysis (PCA)[14,15] represents the objects in a new reference system characterized by new variables called principal components. The first principal component accounts for the maximum variance contained in the original dataset, while subsequent components account for the maximum residual variance. They are calculated hierarchically, so that systematic variations (*i.e.*, information) are explained in the first components while experimental noise and random variations are contained in the last ones. The components are linear combinations of the original variables and are orthogo-
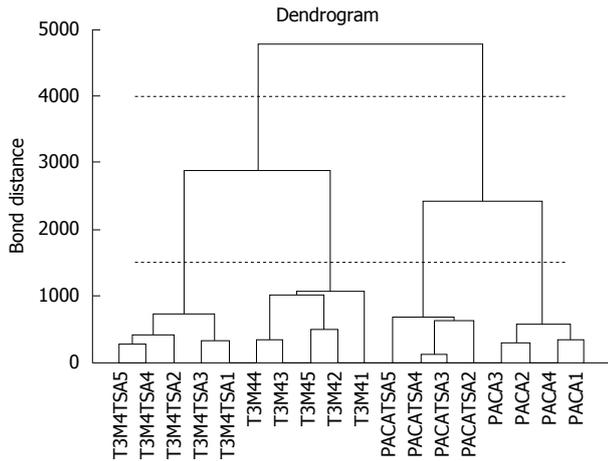
Figure 1 Example of a dendrogram built using Ward's linkage method and euclidean distances. Data refer to samples from two pancreatic cancer cell lines treated or not with trichostatin A.

nal to each other, thus containing independent sources of information. They are often used for dimensionality reduction by considering a smaller number of significant components containing only relevant information.

The graphical representation of the scores (the coordinates of the samples in the new reference system) in the space of the principal components allows the identification of groups of samples showing a similar behavior (samples close one to the other in the graph) or different characteristics (samples far from each other). The corresponding loading plot (representing the loadings, *i.e.*, the coefficients of the linear combination describing each principal component) identifies the variables that are responsible for the analogies or the differences detected for the samples in the score plot.

Cluster analysis

Cluster analysis techniques[14-16] allow the identification of groups of samples or of variables in a dataset by investigating the relationships between the samples or the descriptors. Agglomerative hierarchical methods[14,15] are the most widespread, grouping the samples on the basis of their similarity. The most similar samples or groups are linked first. The final result is a graph, called a dendrogram, where the samples are represented on the X axis and are connected at decreasing levels of similarity along the Y axis. The groups can be identified by applying a horizontal cut of the dendrogram and identifying the number of vertical lines crossed by the horizontal cut. Figure 1 reports a dendrogram where cutting at level 4000 produces only two clusters (the 2 different tumor cell lines), while cutting at level 1500 produces 4 clusters (PACA and T2M4 cell lines, treated and untreated with trichostatin A). The results of hierarchical clustering strongly depend on the measure of similarity and on the linking method adopted. Clustering techniques can be applied to the original variables or to the relevant principal components[16].

Multidimensional scaling

Multidimensional scaling (MDS)[17,18] is aimed at dimensionality reduction and graphical representation of the data. Given a set of *n* objects and a measure of their similarity, MDS searches for a low dimensional space in which the objects are represented by points in the space so that the distances between the points match as much as possible with their original similarities[17]. There are several different approaches to MDS depending on the measure of the similarities matching, on the metrics, on the method used to compute the similarities and on the way the samples configuration is obtained[19,20]. Shepard[21,22] and Kruskal[23] provided an extension of classical MDS to the study of nonparametric similarities.

SUPERVISED CLASSIFICATION METHODS

Classification tools are supervised methods able to separate the objects in the classes present (known a priori, *e.g.*, control *vs* pathological) and provide the variables most responsible for their belonging to different classes (candidate biomarkers). The final aim of their application in the biomedical field is both the development of diagnostic tools and the identification of the differences existing between the classes to shed light on the etiology of a disease or the effect of a new drug. Here, only the methods already applied to the identification of biomarkers for PC will be described.

Soft independent model of class analogy

The soft independent model of class analogy (SIMCA) method[24-27] is based on the independent modelling of each class by means of PCA. Each class is described by its relevant principal components. The samples belonging to each class are contained in the so-called SIMCA boxes, defined by the relevant components of each class. The classification of each sample with SIMCA is not affected by experimental uncertainty and random variations since each class is modelled only by its relevant components. This method is also useful when more variables than objects are available since it performs a substantial dimensionality reduction.

The identification of the candidate biomarkers by SIMCA can be accomplished by the analysis of the discrimination power (DP), a measure of the ability of each variable to discriminate between two classes at a time. The greater the DP, the more a variable weighs on the classification of an object in one of the two classes compared.

Ranking PCA

Ranking PCA is a ranking method proposed by Marengo *et al*[7], Robotti *et al*[28] and successively applied by Polati *et al*[29], based on the description of the original data by means of principal components. The use of PCA in the field of identification of biomarkers in the omics sciences is particularly effective since it allows the relationships be-
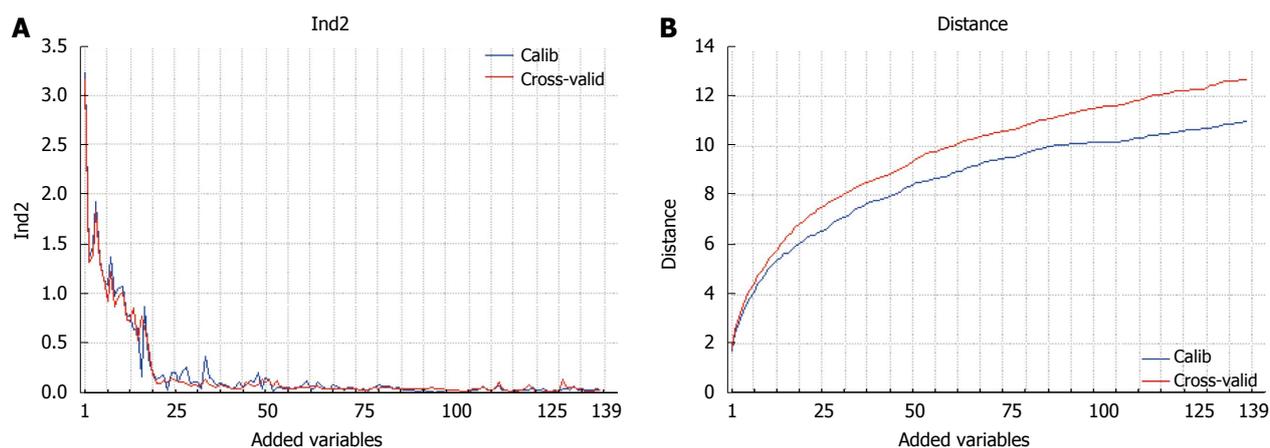
Figure 2 Example of the graphical representation of the results from Ranking-PCA. Trend of Ind_2 *vs* the increasing number of variables added to the model (A); trend of the distance between the two class centroids *vs* the number of variables added to the model (B). Variables on the X-axis are reported in the order in which they are included in the model. Both calibration and cross-validation results are reported.

tween the variables to be taken into consideration, providing sets of correlated biomarkers with a similar function and the possibility of solving problems where the number of variables is larger than the number of samples.

PCA is used here to describe the data coupled to a ranking procedure of the candidate biomarkers in a forward search: one variable is added at each cycle. The first variable selected is the one providing the best separation between the classes on the first principal component. The addition of another discriminating variable further improves the distance between the two classes on the first principal component. If a non-discriminating variable is successively added, the two classes will not be further separated on the same component. Sometimes, more than one component could be necessary for class separation: in this case, different independent sources of information related to the class structure are present in the data and the subsequent principal component accounting for class separation will be included in the model.

The proposed method allows the ranking of the variables according to their discrimination ability, thus assuring the exhaustiveness of the results. The result can be presented in graphical form (Figure 2), where the classification performance or the class distance are reported as a function of the variables added to the model.

Orthogonal partial least squares

Partial least squares (PLS)[14,15,30] establishes a relationship between one or more dependent variables (Y) and a group of descriptors (X). X and Y variables are modeled simultaneously to find the latent variables (LVs) in X that will predict the LVs in Y. These LVs are calculated hierarchically, as for PCA. PLS was originally set up to model continuous responses but it can be applied even for classification purposes (PLS-DA) by establishing an appropriate Y related to the association of each sample to a class. The regression is then carried out between X-block variables and the Y just established. Orthogonal PLS (OPLS)[31] is a modification of PLS developed for

highly decorrelated datasets.

Classification and regression tree

Classification trees[24] are built by subsequent divisions (splits) of subgroups of the original data D in two descending subgroups with the aim of classifying the data in homogeneous groups as much as possible, one different from the others. It is possible to derive a tree diagram where, starting from the root node (where the data D are not separated), a series of nodes and branches separate; each node h represents a subgroup of D. Nodes not undergoing a further split are called terminal nodes: a mode for y is associated with each terminal node.

Starting from the root node h_1, data are separated in a series of splits: in each node, the split giving the most homogeneous division of the data in the two descendent nodes is selected.

Random forests

Random forests[32] is an extension of classification trees and is structured to grow many classification trees. To classify a new object, the new object is first classified by each independent tree in the forest. The forest chooses the most recurrent classification (over all the trees in the forest).

The error rate depends on the correlation between any two trees in the forest (increasing the correlation increases the forest error rate) and the strength of each individual tree in the forest (inversely correlated to the tree error rate).

METHODS FOR THE ANALYSIS OF SURVIVAL OUTCOMES

In clinical trials it is usually important to evaluate the time until the participants present with a particular event (endpoint), *i.e.*, a clinical outcome (death, recurrence of a disease, remission *etc*.). All participants are followed from a certain starting point (operation, starting of a therapy, di-

agnosis, *etc.*) up to the moment when the event occurs (the time requested is recorded). However, often the outcome of some participants is unknown: when the study ends before all participants have presented the event or when some participants withdraw from the study. In these cases (censored data), the time of follow-up is recorded.

Kaplan-Meier estimates of survival functions

The Kaplan-Meier method[33] is used to provide survival functions and can also be effectively applied to censored data. It provides a survival curve where time is reported on the X-axis, while the cumulative survival probability is on the Y-axis. The time corresponding to the point where the curve crosses 50% survival is the estimate of median survival. Kaplan-Meier curves can be compared across groups by mainly applying two non-parametric tests (the log rank test[34] and the generalized Wilcoxon test[35]). The generalized Wilcoxon test[35] is a weighted alternative where early time points weigh more than late ones; this is preferred when the effect of an experimental condition vanishes with time. The log rank test instead is preferred to detect differences during all of the follow-up period.

Cox regression

Cox regression[36] is applied to evaluate the effect of several risk factors on survival, defining the hazard as the probability of the endpoint. The hazard is modeled as:

$$ln\ [H(t)/H_0(t)] = b_1X_1 + b_2X_2 + \cdots + b_kX_k$$

where $H(t)/H_0(t)$ is the so-called hazard ratio (HR), $X_1 \ldots X_k$ are predictor variables and $H_0(t)$ is the baseline hazard at time. In general, the HR may assume only positive values: if it equals 1, the groups show a not statistically different survival; if it is smaller than 1, a subject with a higher value for X has lower risk than a subject with a lower value for X; the opposite behavior is obtained if the HR estimate is larger than 1.

The Cox model works under the so-called proportional hazards assumption: the ratio between the hazards of two patient groups remains constant over the complete follow-up period. Since a HR is calculated in Cox regression, this estimate should apply to all death times: this simplification is only justified if the group difference remains constant over the whole range of follow-up time.

If the monovariate Cox regression is extended to include more than one X variable (multivariate Cox regression), the effect of the interaction between different factors can be evaluated.

OTHER METHODS

Prediction analysis for microarrays

Prediction analysis for microarrays (PAM) performs sample classification from gene expression data and survival outcomes, exploiting the nearest shrunken centroid approach[37]. A standardized centroid for each class is computed. The nearest centroid classification compares the gene expression profile of a new sample to each class centroid: the class whose centroid is closest in squared distance is the predicted class for the new sample.

Nearest shrunken centroid classification[37] is a modification of this approach. When PAM is applied to survival outcomes, supervised principal components analysis[38] is performed, where, instead of using all the genes to perform PCA, only a subset of genes is used, *i.e.*, those highly correlated with survival.

Metropolis algorithm and Monte Carlo simulation

Monte Carlo methods[39] are computational algorithms that rely on repeated random sampling to obtain numerical results; simulations are run several times to calculate probabilities. They are useful for simulating systems with several degrees of freedom and modeling systems characterized by large uncertainty in inputs.

The Metropolis algorithm[40] is used to generate a series of numbers, $X_1, X_2, .., X_n$ with a distribution fixed a priori. The method is based on the generation of numbers that are accepted or rejected to obtain the selected type of distribution.

The Metropolis algorithm can be implemented in Monte Carlo simulations to perform random sampling. The Monte Carlo optimization can be used in biomarkers search to determine the coefficients of model containing the relevant biomarkers.

STUDIES FOR THE IDENTIFICATION OF BIOMARKERS

Studies based on serum and tissue biomarkers determined by non-omics techniques

The studies based on serum and tissue biomarkers determined by non-omics techniques usually exploit monovariate and multivariate Cox regression to evaluate the effect played by different factors on time to progression (TTP) and overall survival (OS). They will be presented here, divided into prognostic/predictive biomarkers and diagnostic biomarkers.

Prognostic and predictive biomarkers

Recent studies about prognostic and/or predictive biomarkers (Table 2) determined in serum or plasma regard the determination of glycoproteins, both alone or associated with other markers, and the determination of circulating factors of the insulin-like growth factor. Three recent studies have been published based on CA19-9, one of the most debated biomarkers for PC. The first study, by Boeck *et al*[41], includes 115 patients with histologically confirmed advanced PC treated with first-line therapy. The novelty of this study is the modelling of the effect of CA 19-9 kinetics by treating it as a time-varying covariate. For CA 19-9 kinetics during chemotherapy, data from 69 patients (TTP) and 84 patients (OS) were available. The proposed approach allowed the modeling of the effect of log (CA 19-9) measured during therapy on

Table 2 Studies based on serum and tissue biomarkers through non-omics techniques

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
41	P	CA 19-9	S	Pretreatment CA 19-9: 115 patients from 5 German centers; 73% treated within prospective clinical trials. Median TTP: 4.4 mo; median OS: 9.4 mo. CA 19-9 kinetics during chemotherapy: 69 patients (TTP) and 84 patients (OS)	Elecsys assay	Cox proportional hazards regression; for CA 19-9 kinetics, CA 19-9 was treated as a time-varying covariate	Univariate analysis: log (CA 19-9) associated with TTP (HR = 1.24; $P < 0.001$) and OS (HR = 1.16; $P = 0.002$). Multivariate analysis: results confirmed. Log(CA 19-9) kinetics during chemotherapy: significant predictor for TTP in univariate analyses (HR = 1.48; $P < 0.001$) and multivariate (HR = 1.45; $P < 0.001$) and for OS (univariate: HR = 1.34; $P < 0.001$; multivariate: HR =1.38; $P < 0.001$)
42	P	CA 19-9, CEA, CRP, LDH and bilirubin	S and P	291 patients; 253 patients (87 %) received treatment within prospective clinical trials. Median TTP: 5.1 mo. Median OS 9.0 mo	Elecsys assay	Kaplan Meier method and Cox proportional hazards regression	Univariate analysis: pre-treatment CA 19-9 (HR = 1.55), LDH (HR = 2.04) and CEA (HR = 1.89) significantly associated with TTP. Baseline CA 19-9 (HR = 1.46), LDH (HR = 2.07), CRP (HR = 1.69) and bilirubin (HR = 1.62) significant prognostic factors for OS. Multivariate analyses: pre-treatment log (CA 19-9) for TTP and log (bilirubin) and log (CRP) for OS had an independent prognostic value
44	P	IGFs	S and P	80 patients received treatment (40 Ganitumab; 40 placebo)	Immunoassays	Kaplan Meier method and Cox proportional hazards regression	Ganitumab associated with improved OS *vs* placebo (HR = 0.49; 95%CI: 0.28-0.87)
45	P	TROP2	T	197 patients; subgroup of 134 patients treated surgically	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	TROP2 overexpression observed in 109 (55%) patients and associated with decreased OS ($P < 0.01$). Univariate Analysis: TROP2 overexpression correlates with lymph node metastasis ($P < 0.04$) and tumor grade ($P < 0.01$). In the subgroup of patients treated surgically, TROP2 overexpression correlated with poor progression-free survival ($P < 0.01$). Multivariate analyses: TROP2 is an independent prognosticator
46	P	JAM-A	T	186 patients; subgroup of 83 patients treated surgically	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	Low expression of JAM-A observed in 79 (42 %) patients and associated with poor OS ($P < 0.01$). Univariate analysis: low expression of JAM-A correlates with positive lymph node status ($P = 0.02$), the presence of distant metastasis ($P = 0.05$), and tumor grade ($P = 0.04$). In the subgroup of patients with surgically resected PC, low expression of JAM-A correlated with decreased progression-free survival ($P < 0.01$). Multivariate analysis: JAM-A was an independent predictor of poor outcome
47	P	TBX4	T	77 stage II PDAC tumors	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	48 cases (62.3%) expressed TBX4 at a high level. No significant correlation between TBX4 expression and other clinicopathological parameters, except tumor grade and liver metastasis recurrence. Survival of patients with TBX4-high expression significantly longer than those with TBX4-low expression ($P = 0.010$). Multivariate analysis: low TBX4 expression independent prognostic factor for OS. TBX4 promoter methylation status frequently observed in PDAC and normal adjacent pancreas
48	P	HSP27	T	86 patients	Tissue microarray (TMA) analysis	Kaplan Meier method and Cox proportional hazards regression	HSP27 expression found in 49% of tumor samples. Univariate analyses: significant correlation between HSP27 expression and survival. Multivariate Cox-regression: HSP27 expression emerged as an independent prognostic factor. HSP27 expression also correlated inversely with nuclear p53 accumulation
49	P	dCK	T	45 patients with resected PDAC received adjuvant gemcitabine based-therapy in multicenter phase 2 studies	Immunohistochemistry	Kaplan Meier method and Cox proportional hazards regression	Median follow-up: 19.95 mo (95%CI: 3.3-107.4 mo). Lymph node (LN) ratio and dCK protein expression significant predictors of DFS and OS in univariate analysis. Multivariate analysis: dCK protein expression the only independent prognostic variable (DFS: HR = 3.48, 95%CI: 1.66-7.31, $P < 0.001$, OS: HR = 3.2, 95%CI: 1.44-7.13, $P < 0.004$)

	Type of marker	Biomarker	Sample	Patients	Method	Statistics	Results
50	P	Notch3 and Hey-1	T	42 patients who underwent resection and 50 patients diagnosed with unresectable PDAC	Immunohistochemistry	Mann-Whitney U test, Wilcoxon test, Cox regression analysis, Kaplan-Meier analysis	All 3 Notch family members significantly elevated in tumor tissue. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1, and HEY-1 (all $P < 0.001$) in locally advanced and metastatic tumors compared to resectable cancers. In survival analyses, nuclear Notch3 and HEY-1 expression significantly associated with reduced OS and DFS following tumor resection with curative intent
51	D and P	21 biomarkers	P	clinically defined cohort of 52 locally advanced (Stage II/III) PDAC cases and 43 age-matched controls	Proximity ligation assay	Combination of the PAM algorithm and logistic regression modeling. Biomarkers that were significantly prognostic for survival were determined using univariate and multivariate Cox survival models	CA19-9, OPN and CHI3L1 were found to have superior sensitivity for pancreatic cancer *vs* CA19-9 alone (93% *vs* 80%). CEA and CA125 have prognostic significance for survival ($P < 0.003$)
52	D	83 circulating proteins	S	333 PDAC patients; 144 controls (benign pancreatic conditions); 227 healthy controls. Samples from each group split randomly into training and blinded validation sets. Panels evaluated in validation set and in patients diagnosed with colon (33), lung (62) and breast (108) cancers	bead-based xMAP immunoassays	A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set	Training set (160 PDAC, 74 Benign, 107 Healthy): panel of CA19-9, ICAM-1, and OPG discriminated PDAC from Healthy controls (SN/SP 88/90%), panel of CA 19-9, CEA, and TIMP-1 discriminated PDAC patients from Benign subjects (SN/SP = 76%/90%). Independent validation set (173 PDAC, 70 Benign, 120 Healthy): panel of CA 19-9, ICAM-1 and OPG demonstrated SN/SP of 78%/94%; panel of CA19-9, CEA, and TIMP-1 demonstrated SN/SP of 71%/89%. The CA19-9, ICAM-1, OPG panel is selective for PDAC and does not recognize breast (SP = 100%), lung (SP = 97%), or colon (SP = 97%) cancer
53	D and P	YKL-40, IL-6, and CA 19.9	P	559 patients with PC from prospective biomarker studies from Denmark ($n = 448$) and Germany ($n = 111$)	ELISA and chemiluminescent immunometric assay	Kaplan Meier method and Cox proportional hazards regression	Odds ratios (ORs) for prediction of PC significant for all biomarkers, with CA 19.9 having the highest AUC (CA 19.9: OR = 2.28, 95%CI: 1.97-2.68, $P = 0.0001$, AUC = 0.94; YKL-40: OR = 4.50, 3.99-5.08, $P = 0.0001$, AUC = 0.87; IL-6: OR = 3.68, 3.08-4.44, $P = 0.0001$, AUC = 0.87). Multivariate Cox analysis: high preoperative IL-6 and CA 19.9 independently associated with short OS (CA 19.9: HR = 2.51, 1.22-5.15, $P = 0.013$; IL-6: HR = 2.03, 1.11-3.70, $P = 0.021$). Multivariate Cox analysis of non-operable patients: high pre-treatment levels of each biomarker independently associated with short OS (YKL-40: HR = 1.30, 1.03-1.64, $P = 0.029$; IL-6: HR = 1.71, 1.33-2.20, $P = 0.0001$; CA 19.9: HR = 1.54, 1.06-2.24, $P = 0.022$). Patients with preoperative elevation of IL-6 and CA 19.9 had shorter OS ($P = 0.005$) compared to patients with normal levels (45% *vs* 92% alive after 12 mo)

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample: S: Serum; P: Plasma; T: Tissue; TTP: Time to progression.

the event (TTP or OS) rather than modeling the effect of the pretreatment value of log (CA 19-9).

In the second study, Haas *et al*[42] pooled pre-treatment data on CA 19-9, carcinoembryonic antigen (CEA), C-reactive protein (CRP), lactate dehydrogenase (LDH) and bilirubin from two multicenter randomized phase II trials and prospective patient data. Marker levels were assessed before the start of palliative first-line therapy for advanced PC and during treatment (for CA 19-9 only).

In the third study, Boeck *et al*[43] evaluated pre-treatment (palliative first-line chemotherapy) values and weekly values of cytokeratin 19-fragments (CYFRA 21-1), CA 19-9 and CEA in blood samples from patients with PC. CYFRA 21-1 are biomarkers for different epithelial diseases but their role in PC has not been investigated yet.

In Boeck *et al*[41] pre-treatment log (CA19-9) proved to be significantly associated with TTP and OS. Moreover, log (CA 19-9) kinetics after the start of treatment was found to be a significant predictor for both TTP and OS. Similar results were found by Haas *et al*[42] where pre-treatment CA 19-9, LDH and CEA levels were significantly associated

with TTP. Regarding OS, baseline CA 19-9, LDH, CRP and bilirubin were significant. Boeck *et al*[43] found that CYFRA 21-1 and CA 19-9 showed a high correlation with TTP and OS, while in multivariate analysis, only CYFRA 21-1 and performance status were independent predictors for OS.

McCaffery *et al*[44] assessed the predictive nature of baseline circulating factors of the insulin-like growth factor (IGF) axis on the treatment effect of ganitumab plus gemcitabine in metastatic PDAC. Baseline levels of IGFs/IGF binding proteins were analyzed in serum or plasma while mutations and gene expression were analyzed in archival samples. Ganitumab was associated with improved OS *vs* placebo. The treatment effect on improved OS was larger in patients with higher levels of IGF-1, IGF-2 or IGFBP-3, or lower levels of IGFBP-2. Interaction between treatment and IGFs/IGFBPs showed predictive potential for IGF-2 and IGFBP-2.

The studies about prognostic and/or predictive biomarkers determined in tissue samples, usually by immunohistochemistry or tissue microarray analysis (TMA), instead include the determination of glycoproteins, other proteins and enzymes.

Fong *et al*[45] investigated the expression of TROP2 (human trophoblast cell-surface) antigen, a glycoprotein found to be strongly expressed in a variety of human epithelial cancers, and the expression of junctional adhesion molecule A (JAM-A) antigen[46], a type I transmembrane glycoprotein, which has been recently shown to affect the prognosis of several malignancies. The two studies involved 197 and 186 patients with PDAC respectively. TROP2 overexpression was observed in 55% of patients, while low expression of JAM-A was observed in 42% of samples; both markers were significantly associated with decreased OS. They were both correlated with lymph node metastasis and tumor grade. In the subgroup of patients surgically treated with curative intent, TROP2 and low expression of JAM-A correlated with poor progression-free survival.

In the study by Zong *et al*[47], the expression of the T-box transcription factor 4 (TBX4) was investigated in 77 stage II PDAC tumors. 62.3% of cases expressed TBX4 at a high level. Significant correlation was only detected between TBX4 expression and tumor grade and liver metastasis recurrence. The survival with TBX4-high expression was significantly longer.

Applying tissue microarray (TMA) analysis, Schäfer *et al*[48] correlated heat shock protein 27 (HSP27) expression status with clinicopathological parameters in PDAC from 86 patients. HSP27 expression was found in 49% of tumor samples. A significant correlation was found with OS. The authors also assessed the impact of HSP27 on chemo- and radio-sensitivity directly in PC cells. HSP27 expression emerged as an independent prognostic factor and correlated inversely with nuclear p53 accumulation, indicating protein interactions between HSP27 and p53 or TP53 mutation-dependent HSP27-regulation. HSP27 overexpression rendered HSP27 low-expressing PL5 PC

cells more susceptible to treatment with gemcitabine, while HSP27 protein depletion in HSP27 high-expressing AsPC-1 cells caused increased gemcitabine resistance.

Maréchal *et al*[49] identified deoxycytidine kinase (dCK), a recombinant enzyme, to be associated with prolonged survival after adjuvant gemcitabine administration for resected PDAC. The study involved 45 patients. The lymph node (LN) ratio and dCK protein expression were significant predictors of DFS and OS in univariate analysis. On multivariate analysis, a step-down procedure based on the likelihood ratio test, dCK protein expression was the only independent prognostic factor.

Having previously reported that Notch3 activation appeared to be associated with more aggressive PC disease, Mann *et al*[50] examined components of this pathway (Notch1, Notch3, Notch4, HES-1, HEY-1) in resectable and non-resectable tumors compared to uninvolved pancreas. All three Notch family members were significantly increased in tumor tissue, with expression maintained within matched lymph node metastases. Significantly higher nuclear expression of Notch1, -3 and -4, HES-1 and HEY-1 was noted in locally advanced and metastatic tumors compared to resectable cancers. Nuclear Notch3 and HEY-1 expression were significantly associated with reduced OS and DFS following tumor resection.

Diagnostic biomarkers

Regarding diagnostic biomarkers (Table 2), the most recent studies are based on the determination of protein panels in serum or plasma, exploiting ELISA or proximity ligation assay (PLA) for their determination. PLA is a highly sensitive technique for multiplex detection of biomarkers in plasma with little interfering background signal. Some of the studies proposed the determination of both diagnostic and prognostic markers. All studies presented here involve CA19-9 as a potential biomarker in association with other markers.

In the first study, Chang *et al*[51] applied PLA to the identification of plasma levels of 21 biomarkers in 52 locally advanced PDAC cases and 43 age-matched controls. The optimal diagnostic biomarker panel was computed using a combination of the PAM algorithm and logistic regression modeling.

In the second study, Brand *et al*[52] investigated 83 circulating proteins in sera of patients diagnosed with PDAC, benign pancreatic conditions and healthy controls. Samples from each group were split randomly into training and blinded validation sets prior to analysis. A Metropolis algorithm with Monte Carlo simulation (MMC) was used to identify discriminatory biomarker panels in the training set. Identified panels were evaluated in the validation set and in patients diagnosed with colon, lung and breast cancers.

In the third study, Schultz *et al*[53] tested the hypothesis that high plasma YKL-40 and IL-6 are associated with PC and short OS. 559 patients with PC from prospective biomarker studies were studied. Plasma YKL-40 and IL-6 were determined by ELISA and serum CA 19.9 by che-

miluminescent immunoassay.

Chang *et al*[51] found that three markers (CA19-9, OPN and CHI3L1) have superior sensitivity for PC *vs* CA19-9 alone (93% *vs* 80%) and two markers (CEA and CA125) proved to have a prognostic significance for survival of PC ($P < 0.003$) when measured simultaneously. Brand *et al*[52] found that the panel of CA 19-9, CEA and TIMP-1 discriminated PDAC patients from benign subjects with an SN/SP of 76%/90% in the training set and of 71%/89% in the validation set. The CA19-9, intercellular adhesion molecule 1 (ICAM-1), OPG panel is selective for PDAC and does not recognize breast (SP = 100%), lung (SP = 97%) or colon (SP = 97%) cancer. Schultz *et al*[53] instead showed that high preoperative IL-6 and CA 19.9 were independently associated with short OS. High pre-treatment levels of each biomarker were independently associated with short OS in non-operable patients.

PROTEOMIC BASED STUDIES

The most recent studies on identification of biomarkers in proteomics (Table 3) are mainly regarding the determination of diagnostic markers. The studies are presented separating those carried out from serum or tissue samples and those carried out on cell lines or animal models. The studies presented here are mainly devoted to identifying exploratory biomarkers.

The studies based on proteomic approaches show a great potential for the identification of PC biomarkers; the panels of markers identified by these techniques are characterized by good performance regarding both sensitivity and specificity, showing results at least equal to or in some cases better than classical approaches. It is the authors' opinion that, in future, the information provided by such high-throughput techniques should be coupled with clinical information to provide exhaustive sets of biomarkers with better predictive ability.

Diagnostic biomarkers in tissue and serum samples

Tissue and serum biomarkers in proteomics are usually determined by: SDS-PAGE followed by LC-MS for identifying the most up- or down-regulated proteins; matrix-assisted laser desorption ionization time-of-flight (MAL-DI-TOF) mass spectrometry; and surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry. The exploitation of instrumental techniques providing a high amount of information makes the application of multivariate methods necessary in order to detect panels of biomarkers with the best predictive ability.

In the first study by McKinney *et al*[54], matched pairs of tumor and non-tumor pancreas from patients undergoing tumor resection were treated to obtain cytosol, membrane, nucleus and cytoskeleton cellular protein fractions. The fractions were analyzed by SDS-PAGE followed by LC-MS/MS to identify 2393 unique proteins. The spectral count data were compared using a power law global error model (PLGEM) to identify statistically significant protein changes between non-tumor and tumor samples[55,56]. Among the 104 proteins significantly changed in cancers, four (biglycan (BGN), pigment epithelium-derived factor (PEDF), thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3)) were further validated and proved to be up-regulated in cancer and have potential for development as minimally-invasive diagnostic markers.

Kojima *et al*[57] differentiated pancreatic neoplasia from non-neoplastic pancreatic disease. Samples from 50 patients [15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP)] were collected. A high-throughput method was applied, using high-affinity solid lipophilic extraction resins, enriched low molecular weight proteins for extraction with a high-speed MALDI-MS. Multivariate analysis was carried out by MDS as in Mobley *et al*[58]. Using eight serum features, Ca were differentiated from H (SN = 88%, SP = 93%), Ca from CP (SN = 88%, SP = 30%) and Ca from both H and CP combined (SN = 88%, SP = 66%). In addition, nine features obtained from urine differentiated Ca from both H and CP, combined with high efficiency (SN = 90%, SP = 90%). Interestingly, the plasma samples did not show significant differences.

Ehmann *et al*[59] and Hauskrecht *et al*[60] instead applied SELDI-TOF mass spectrometry. In the first study[59], 96 serum samples from patients undergoing cancer surgery were compared with 96 controls. Samples were fractionated by anion exchange chromatography. Data analysis, involving Mann-Whitney *U* test and classification and regression tree (CART) analysis, identified 24 differentially expressed protein peaks, 21 of which were underexpressed in cancer samples. The best single marker can predict 92% of controls and 89% of cancer samples. The best model with a set of 3 markers showed a sensitivity of 100% and a specificity of 98% for the training data and a sensitivity of 83% and a specificity of 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I were identified as markers (decreased in cancer sera). Hauskrecht *et al*[60] instead proposed a feature selection method that extracts useful feature panels from high-throughput spectra. 57 PC samples were compared to 59 controls. The results clearly show the improved classification performances when the method is compared to standard strategies.

The last study makes use of protein microarrays to explore whether a humoral response to PC-specific tumor antigens has utility as a biomarker of PC. To determine if such arrays can be used to identify novel autoantibodies in the sera from PC patients, Patwa *et al*[61] resolved proteins from a PDAC cell line (MIAPACA) by 2-D liquid-based separations and then arrayed them on nitrocellulose slides. The slides were probed with sera from a set of patients diagnosed with PC and compared with age- and sex-matched normal subjects. To account for patient-to-patient variability, a non-parametric Wilcoxon rank-sum test was used in which protein biomarkers were identified. Classification by the PAM algorithm showed 86.7% accuracy, with a SN and SP of 93.3% and 80%, respectively. The identified candidate autoantibody

Table 3 Proteomic based studies

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
54	D	Among 2393 unique proteins, 104 proteins significantly changed in cancer	T	5 patients; matched pairs of tumor and non-tumor pancreas	Tissues treated to obtain cytosol, membrane, nucleus and cytoskeletonfractions. Fractions separated and digested underwent LC-MS/MS	PLGEM	104 proteins significantly changed in cancer. Among these, 4 proteins validated that were up-regulated in cancer: biglycan (BGN), Pigment Epithelium-derived Factor (PEDF) Thrombospondin-2 (THBS-2) and TGF-β induced protein ig-h3 precursor (βIGH3)
57	D	Serum MALDI-TOF features	S	15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP) samples	MALDI-TOF	Nonparametric	8 serum features: Ca samples differentiated from H (SN = 88%, SP = 93%), Ca from CP (SN = 88%, SP = 30%), and Ca from both H and CP combined (SN = 88%, SP = 66%). 9 features obtained from urine: differentiated Ca from both H and CP combined (SN = 90%, SP = 90%)
59	D	Serum SELDI-TOF features	S	96 serum samples from patients undergoing cancer surgery compared with sera from 96 controls	SELDI-TOF	pairwise statistics, MDS, hierarchical analysis Mann-Whitney *U* test, CART	Data analysis identified 24 differentially expressed protein peaks, 21 of which under-expressed in cancer samples. The best single marker predicts 92% of controls and 89% of cancer samples. Multivariate analysis: best model (3 markers) with SN = 100% and SP = 98% for the training data and SN = 83% and SP = 77% for test data. Apolipoprotein A-II, transthyretin and apolipoprotein A-I identified as markers and decreased at least 2 fold in cancer sera
60	D	Serum SELDI-TOF features	S	57 PC samples were compared to 59 controls	SELDI-TOF	Multivariate decorrelation filtering	Improved classification performances when the presented strategy is compared to standard univariate feature selection strategies
61	D	Proteins	S	Sera from patients diagnosed with PC compared with age- and sex-matched normal subjects	Protein microarrays	Rank-based non-parametric statistical testing	A serum diagnosis of PC was predicted with 86.7% accuracy, with a sensitivity and specificity of 93.3% and 80%. Candidate autoantibody biomarkers studied for their classification power using an independent sample set of 238 sera. Phosphoglycerate kinase-1 and histone H4 noted to elicit a significant differential humoral response in cancer sera compared with age- and sex-matched sera from normal patients and patients with chronic pancreatitis and diabetes
62	D	Proteins	PDAC cell lines	435 spots identified from 18 samples from 2 cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells	2D-PAGE	PCA, SIMCA, Ranking-PCA	Samples were all perfectly classified. Significant proteins were further identified by MS analysis
63	D	Proteins regulating the conversion of quiescent to activated PaSC cells	rat PaSC cell line	-	SDS-PAGE and GeLC-MS/MS	QSPEC	Qualitative and quantitative proteomic analysis revealed several hundred proteins as differentially abundant between the two cell states. Proteins of greater abundance in activated PaSC: isoforms of actin and ribosomal proteins. Proteins more abundant in non-proliferating PaSC: signaling proteins MAP kinase 3 and Ras-related proteins

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample; S: Serum; P: Plasma; T: Tissue.

biomarkers were validated using an independent sample set of 238 samples. Phosphoglycerate kinase-1 and histone H4 were noted to elicit a significant differential humoral response in cancer sera.

Diagnostic biomarkers from cell lines or animal model samples

Diagnostic biomarkers in proteomics have also been recently determined from cell lines (PACA44, T3M4) or on animal models. The most exploited analytical techniques in this case are SDS-PAGE, followed by LC-MS, 2D-PAGE or 2D-LC approaches. Marengo *et al*[62] identified the regulatory proteins in human PC treated with trichostatin A by 2D-PAGE maps and multivariate analysis. PCA was applied to a spot quantity

dataset comprising 435 spots detected in 18 samples belonging to two different cell lines (Paca44 and T3M4) of control and drug-treated PDAC cells. PCA allowed the identification of the groups of samples present in the dataset; the loadings analysis allowed the identification of the differentially expressed spots, which characterize each group of samples. The treatment of both the cell lines with trichostatin A showed an evident effect on the proteomic pattern of the treated samples. Identification of some of the most relevant spots was also performed by MS analysis. The same authors applied different multivariate statistical tools to the same set of data to provide sets of candidate biomarkers; the first application regards the exploitation of SIMCA classification to evaluate the biomarkers characterized by a significant discriminant power[25], while the second application regards the development of ranking PCA[28]. This second application is particularly interesting for overcoming the limitations of the methods usually adopted as variable selection tools to identify only significant biomarkers: they are usually aimed at the selection of the smallest set of variables (spots) providing the best performances in prediction. This approach does not seems to be the best choice in the identification of potential biomarkers since all the possible candidate biomarkers have to be identified to provide a general picture of the "pathological state"; exhaustivity has to be preferred to provide a complete understanding of the mechanisms underlying the pathology. Ranking PCA allowed the exhaustive identification of a complete set of candidate biomarkers.

Paulo *et al*[63] compared differentially expressed proteins in rat in activated and serum-starved non-proliferating pancreatic stellate cells (PaSC), emerging key mediators in chronic pancreatitis and PC pathogenesis. About 1500 proteins were identified after SDS-PAGE and LC-MS/MS. Qualitative and quantitative proteomic analysis revealed several hundred proteins to be differentially abundant between the two cell states. Significance analysis was performed using QSPEC, a recently published algorithm for determining the statistical significance of differences in spectral counting data from two sample sets[64]. This algorithm exploits the Bayes factor instead of the *P*-value as a measure of statistical significance[65,66]. Proteins of greater abundance in activated PaSC included isoforms of actin (*e.g.,* smooth muscle actin) and ribosomal proteins. Proteins more abundant in non-proliferating PaSC than in activated PaSC included signaling protein MAPK-3 and Ras-related proteins. The molecular functions and biological pathways for these proteins were also determined by gene ontology analysis and KEGG pathway.

Other studies based on a proteomic approach

Paulo *et al*[67] also evaluated the endoscopic pancreatic function test (ePFT) as a method able to safely obtain pancreatic fluid for MS analysis from patients during upper endoscopy and reproducibly identify pancreas-specif-

ic proteins. The ePFT-collected pancreatic fluid from 3 individuals without evidence of chronic pancreatitis was analyzed by SDS-PAGE and GeLC-MS/MS. The SDS-PAGE analysis revealed no significant variation in protein concentration during the 1 h collection. The GeLC-MS/MS analysis identified pancreas-specific proteins previously described from endoscopic retrograde cholangio-pancreatography and surgical collection methods. Gene ontology further revealed that most of the proteins identified have a molecular function of proteases.

GENOMIC-BASED STUDIES

The most recent studies on identification of biomarkers in genomics (Table 4) regard both the determination of prognostic/predictive markers and diagnostic markers and are presented hereafter separated into these two classes. The studies presented here are mainly devoted to identifying exploratory biomarkers.

The studies based on genomic approaches have potential to identify PC biomarkers. In future, the information provided by genomic approaches should be coupled to proteomic, metabolomic and clinical information to improve the predictive ability of the panels of identified biomarkers.

Prognostic and/or predictive biomarkers

Prognostic and predictive biomarkers in genomics (Table 4) are usually determined in tissue samples. Some of the proposed studies include both protein expression and microRNA expression profiles. In these studies, multivariate Cox regression analysis is usually exploited.

Ogura *et al*[68] studied the K-ras mutation status in 242 patients with unresectable PC. The authors focussed on K-ras mutation subtypes since recent reports indicate that K-ras mutation status acts as a prognostic factor. CA19-9, metastatic stage and mutant-K-ras were negative prognostic factors, indicating a reduced survival. Among the patients who had K-ras mutation subtypes, CA19-9, metastatic stage and the presence of the G12D or G12R mutations were negative prognostic factors for OS.

Hwang *et al*[69] evaluated whether expression of novel candidate biomarkers, including microRNAs, can predict clinical outcome in PDAC patients treated with adjuvant therapy. 82 resected PDAC cases were analyzed for protein expression by immunohistochemistry and for microRNA expression by quantitative real time PCR (qRT-PCR). Lower than median miR-21 expression was associated with a significantly lower HR for death and recurrence in the subgroup of patients treated with adjuvant therapy. MiR-21 expression status emerged as the single most predictive biomarker for treatment outcome. No significant association was detected in patients not treated with adjuvant therapy. The results were confirmed in an independent validation of 45 PDAC tissues.

One-fifth of patients with seemingly "curable" PDAC experienced an early recurrence and death, while

Table 4 Genomic based studies

Ref.	Type of marker	Markers	Sample	Study group	Analytical methods	Statistical methods	Performance
68	P	K-ras mutation status and subtypes	endoscopic ultrasound-guided fine-needle aspiration specimens	242 patients	RT-PCR	Kaplan Meier method and Cox proportional hazards regression	Multivariate analysis: CA19-9 C 1000 U/mL (HR = 1.78, 95 %CI: 1.28-2.46, $P < 0.01$), metastatic stage (HR 2.26, 95 % CI 1.58-3.24, $P < 0.01$) and mutant-K-ras (HR 1.76, 95 %CI: 1.03-3.01, $P = 0.04$) negative prognostic factors. Among patients with K-ras mutation subtypes: CA19-9 C 1000 U/mL (HR 1.65, 95%CI: 1.12-2.37, $P < 0.01$), metastatic stage (HR 2.12, 95%CI: 1.44-3.14, $P < 0.01$), and G12D or G12R mutations (HR = 1.60, 95%CI:1.11-2.28) negative prognostic factors for OS
69	P	MicroRNA-21	T	82 resected Korean PDAC cases. Subgroup of patients treated with adjuvant therapy ($n = 52$)	Protein expression by immunohistochemistry microRNA expression by qRT-PCR	Cox proportional hazards model	Subgroup with adjuvant therapy: lower than median miR-21 expression associated with lower HR for death (HR = 0.316, 95%CI = 0.166-0.600, $P = 0.0004$) and recurrence (HR = 0.521, 95%CI = 0.280-0.967, $P = 0.04$). MiR-21: single most predictive biomarker for treatment outcome. No significant association in patients not treated with adjuvant therapy. Independent validation cohort of 45 frozen PDAC tissues from Italian cases treated with adjuvant therapy: lower than median miR-21 expression confirmed to be correlated with longer OS and DFS
71	P	13 putative PDA biomarkers from the public biomarker repository		A survival tissue microarray was constructed comprised of short-term (cancer-specific death, 12 mo, $n = 58$) and long-term survivors (30 mo, $n = 79$) who underwent resection for PDA (total, $n = 137$)	Immunohistochemical analyses; survival tissue microarray (s-TMA)	Wilcoxon rank sum test	Multivariate model: MUC1 (OR = 28.95, 3+ *vs* negative expression, $P = 0.004$) and MSLN (OR = 12.47, 3+ *vs* negative expression, $P = 0.01$) highly predictive of early cancer-specific death. Pathological factors (size, lymph node metastases, resection margin status, and grade); ORs < 3 and none reached statistical significance. ROC curves used to compare the 4 pathological prognostic features (ROC area = 0.70) to 3 univariate molecular predictors (MUC1, MSLN, MUC2) of survival group (ROC area = 0.80, $P = 0.07$)
1	P	MTA2 mRNA and protein expression	T	123 PDAC samples and 40 control tissues	qRT-PCR and immunohistochemistry	Kaplan-Meyer curves and Cox analysis	MTA2 mRNA and protein expression levels up-regulated in PC. MTA2 correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS. MTA2: independent prognostic factor.
72	D	Leukocyte DNA Methylation	Blood	Phase I: 132 never-smoker PaC patients and 60 never-smoker healthy controls. Phase II : validation of 88 of 96 phase I selected CpG sites in 240 PaC cases and 240 matched controls	DNA array	Wilcoxon Rank Sum tests and likelihood penalized logistic regression models	Significant differences found in 110 CpG sites (FDR < 0.05). Phase II: 88 of 96 phase I selected CpG sites validated in 240 PaC cases and 240 matched controls ($P <$ 0.05). Prediction model: 5 CpG sites (IL10_P348, LCN2_P86, ZAP70_P220, AIM2_P624, TAL1_P817) discriminated PaC from controls (C = 0.85 in phase I; 0.76 in phase II). One CpG site (LCN2_P86) could discriminate resectable patients from controls (C = 0.78 in phase I; 0.74 in phase II). 3 CpG sites identified (AGXT_P180_F, ALOX12_E85_R, JAK3_P1075_R) where the methylation levels were significantly associated with SNPs (FDR < 0.05)
73	D	cell-surface targets	T	28 PC specimens and 4 normal pancreas tissue samples. Expression in normal tissues evaluated by array data from 103 samples representing 28 organ sites as well as mining published data	Complementary assays of mRNA expression. Immunohistochemistry. qRT-PCR	-	170 unique targets highly expressed in 2 or more PC specimens and not expressed in normal pancreas samples. Two targets (TLR2 and ABCC3) further validated for protein expression by tissue microarray based immunohistochemistry have potential for the development of diagnostic imaging and therapeutic agents for PC
74	D	Differentially expressed genes	Blood	25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 yr, and 25 healthy controls. Results further validated for 101 blood samples	Microarray and qRT-PCR	-	58 genes found to be unique in patients with cancer-associated diabetes, including 23 up-regulated and 35 down-regulated genes. 11 up-regulated genes further validated by RT-PCR; 2 of these (VNN1 and MMP9) selected for logistic regression analysis. The combination of VNN1 and MMP9 showed the best discrimination of cancer-associated diabetes from type 2 diabetes. The protein expression of MMP9 and VNN1 was in accordance with the gene expression

Type of marker: P: Prognostic/predictive; D: Diagnostic; Sample: S: Serum; P: Plasma; T: Tissue; PDAC: Pancreatic ductal adenocarcinoma; VNN1: Vanin-1; MMP9: Matrix metalloproteinase 9.

some patients with advanced stage tumors are deemed "unresectable" by conventional staging criteria, yet progress slowly. Effective biomarkers that stratify PDAC based on the biological behavior are therefore needed. Building on a compendium of 2500 published candidate biomarkers in PDAC[70], Winter *et al*[71] constructed a survival tissue microarray (s-TMA) comprised of short-term (12 mo) and long-term survivors (30 mo) who underwent resection for PDAC. The s-TMA acts as a biological filter to identify prognostic markers. 13 putative PDAC biomarkers were identified from the public biomarker repository and tested against the s-TMA. MUC1 and MSLN were highly predictive of early cancer-specific death. By comparison, no pathological factors (size, lymph node metastases, resection margin status and grade) reached statistical significance.

Chen *et al*[1] detected metastasis-associated gene 2 (MTA2) expression in PDAC and related it to prognosis. MTA2 mRNA and protein expression were determined by qRT-PCR and immunohistochemistry in primary cancers and their adjacent non-cancerous tissues. MTA2 mRNA and protein expression levels were up-regulated in PC. MTA2 was correlated with poor tumor differentiation, TNM stage and lymph node metastasis. Patients with high expression levels of MTA2 showed lower OS.

Diagnostic biomarkers

Diagnostic biomarkers from genomics-based studies (Table 4) are identified both from tissue and serum samples.

To identify biomarkers for early detection, Pedersen *et al*[72] examined DNA methylation differences in leukocyte DNA between PC cases and controls. In phase I, methylation levels were measured at 1505 CpG sites in leukocyte DNA from 132 never-smoker PC patients and 60 never-smoker controls. Significant differences were found in 110 CpG sites. In phase II, 88 of 96 phase I selected CpG sites were tested and validated in 240 PC cases and 240 matched controls. Using penalized logistic regression, a prediction model was built consisting of five CpG sites (IL10_P348, LCN2_P86, ZAP70_P220, AIM2_P624, TAL1_P817) that discriminated cancer patients from controls. One CpG site (LCN2_P86) alone could discriminate resectable patients from controls.

Morse *et al*[73] used complementary assays of mRNA expression profiling of cell-surface genes to determine increased expression in PC *vs* normal pancreas tissues and validated protein expression by immunohistochemistry on tissue microarrays. This approach was aimed at the identification of targets for potential use in the molecular imaging of cancer, allowing for non-invasive determination of tumor therapeutic response and molecular characterization of the disease, or in the targeted delivery of therapy to tumor cells, decreasing systemic effects. Expression profiles of 2177 cell-surface genes for 28 pancreatic tumor specimens and 4 controls were evaluated. 170 unique targets were highly expressed in 2 or more of the pancreatic tumor specimens and were not expressed in controls. Two targets (TLR2 and ABCC3)

were further validated for protein expression and proved to be potential for the development of diagnostic imaging and therapeutic agents for PC.

Huang *et al*[74] explored specific biomarkers that can differentiate PC-associated diabetes from type 2 diabetes for the early detection of PC. Peripheral blood samples were collected from 25 patients diagnosed with PC and diabetes, 27 patients with PC without diabetes, 25 patients with diabetes mellitus > 5 years, and 25 controls. 32 samples were used in microarray experiments to find differentially expressed genes specific for cancer-associated diabetes. The results were further validated by quantitative qRT-PCR for 101 blood samples. Protein expression of selected genes in serum and tissues was also detected. 58 genes were found to be unique in patients with cancer-associated diabetes (23 up-regulated; 35 down-regulated). 11 up-regulated genes were further validated by RT-PCR and 2 of these, vanin-1 (*VNN1*) and matrix metalloproteinase 9 (*MMP9*), showed the best discrimination of cancer-associated from type 2 diabetes.

METABOLOMICS BASED STUDIES

Just one paper, by Kaur *et al*[75], has recently appeared in the literature, reporting for the first time the mass spectrometry-based metabolomic profiling of human pancreas in matched tumor and normal tissues. UPLC coupled with TOF-MS was applied to perform small molecule metabolite profiling of matched normal and PC tissues. The resulting multivariate data matrix was pre-processed for spectral alignment and peak detection, followed by normalization of the data to the feature intensities of the internal standard as well as to the total protein concentration. The normalized data were analyzed first by PCA, followed by OPLS[31]. The authors also exploited random forest clustering[32] to interrogate the top 50 features with significant alterations in the tumor tissue compared to the control. The candidate markers were searched against different databases[76,77] to find compounds that corresponded to the accurate monoisotopic mass measurements detected by UPLC-TOFMS analysis. The authors report a subset of metabolites which were unequivocally identified and found to be significantly de-regulated in PC tissues.

The study reported here proves that metabolomic profiling shows great potential for the identification of biomarkers for PC. Certainly, further characterization and validation with a large sample size is needed and may help establish the utility of such markers as biomarkers of clinical benefit.

CONCLUSION

This review aimed to present the most recent applications of the omics approaches (proteomics, genomics and metabolomics) to the identification of biomarkers for PC. Particular attention has been paid to the statistical methods adopted for identification of biomarkers, first

presenting the main statistical procedures adopted from a theoretical point of view. Then, the most recent applications present in the literature were presented separately for non-omic, proteomic, genomic and metabolomic based studies. Within this distinction, studies were presented separately for diagnostic and prognostic/predictive biomarkers and according to the type of marker.

Different statistical approaches are exploited in the literature for the identification of markers in PC; the methodologies presented here appear to be effective and sound. However, it is the authors' opinion that multivariate methods have to be preferred. With the term multivariate, the authors refer to methods evaluating the relationships between the variables (both predictors and outcomes if several of both are present) in order to provide a pool of markers highlighting synergistic and antagonistic effects. In fact, the biological effect played by pathology (and PC makes no exception) is the result of a series of different mechanisms independent from each other or showing relevant interactions. Among all strategies, therefore, multivariate ones able to point out these relationships are preferred.

Another hint that must be addressed is the risk of identifying false positives, *i.e.*, markers erroneously identified as such; this risk greatly increases when little information is available (*i.e.*, a small number of cases/patients is investigated). Certainly, this problem is deeply related to the problem of experimental design and sample collection and each study should be carefully designed from a statistical point of view before being performed in order to include all possible sources of biological variation. Of course, this necessity often clashes with the availability of samples, especially when tissue collection is involved. From a statistical point of view, in these cases characterized by little information, it is very important to apply mathematical tools to validate the models built, thus evaluating the predictive ability of the models. In this respect, the use of cross-validation techniques or simulation algorithms is fundamental to identify only statistically significant markers.

Certainly, there is a great gap between the results presented in studies on the identification of candidate markers reported in literature and the actual possibility of exploiting the identified biomarkers at a clinical level. This is due to several aspects, among which the most important are the poor sensitivity/specificity sometimes characterizing the identified pools of markers and the complicated biostatistic design of prospective studies for their validation prior to clinical use.

It is the authors' opinion that the future perspective in exploratory identification of biomarkers has to be found in the exhaustive search for potential markers. It is impossible to imagine that complex pathology acting on a wide range of individuals characterized by a large biological variability could be reflected in a very restricted panel of markers. We think that the future will rely on high-throughput techniques and the possibility of combining the results emerging from proteomic, genomic, metabo-

lomic studies coupled with clinical information to identify exhaustive panels of markers, thus improving the predictive performance of the panels themselves and providing better sensitivity and specificity. Certainly, great attention has to be paid in such studies to the proper evaluation of experimental and biological variability (*i.e.*, a careful selection of experimental design) to provide sound and robust results and to the evaluation of the results through effective multivariate techniques.

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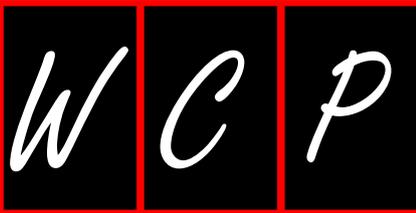
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Gene therapy in pancreatic cancer

Si-Xue Liu, Zhong-Sheng Xia, Ying-Qiang Zhong

Si-Xue Liu, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China

Zhong-Sheng Xia, Ying-Qiang Zhong, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Guangdong Province, China

Author contributions: Liu SX performed the research; Liu SX and Xia ZS analyzed the data; Liu SX and Zhong YQ wrote the review.

Correspondence to: Ying-Qiang Zhong, MD, PhD, Professor, Department of Gastroenterology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, Yanjiang West Road 107, Guangdong Province, China. zhongyingqiang@21cn.com

Telephone: +86-20-81332598 Fax: +86-20-81332244

Abstract

Pancreatic cancer (PC) is a highly lethal disease and notoriously difficult to treat. Only a small proportion of PC patients are eligible for surgical resection, whilst conventional chemoradiotherapy only has a modest effect with substantial toxicity. Gene therapy has become a new widely investigated therapeutic approach for PC. This article reviews the basic rationale, gene delivery methods, therapeutic targets and developments of laboratory research and clinical trials in gene therapy of PC by searching the literature published in English using the PubMed database and analyzing clinical trials registered on the Gene Therapy Clinical Trials Worldwide website (http://www. wiley.co.uk/genmed/ clinical). Viral vectors are main gene delivery tools in gene therapy of cancer, and especially, oncolytic virus shows brighter prospect due to its tumor-targeting property. Efficient therapeutic targets for gene therapy include tumor suppressor gene *p53*, mutant oncogene *K-ras*, anti-angiogenesis gene *VEGFR*, suicide gene *HSK-TK*, cytosine deaminase and cytochrome *p450*, multiple cytokine genes and so on. Combining different targets or combination strategies with traditional chemoradiother- apy may be a more effective approach to improve the efficacy of cancer gene therapy. Cancer gene therapy is not yet applied in clinical practice, but basic and clinical studies have demonstrated its safety and clinical benefits. Gene therapy will be a new and promising field for the treatment of PC.

Core tip: This paper tries to present a full picture of gene therapy in pancreatic cancer, providing an unambiguous classification and comprehensive analysis, especially in therapeutic targets and clinical trials worldwide. From our work, you may find the hotspots in related research and the reason why they get there.

INTRODUCTION

Pancreatic cancer (PC) is an aggressive and highly lethal malignant disease. The incidence of PC is lower than that of many other types of cancer, but it is the fourth most common cause of death from cancer[1]. PC is highly malignant and invasive, owing to nonspecific incipient symptoms and early metastasis. Most patients have local or metastatic spread at the time of presentation, and less than 15% of patients are candidates for surgery. Therefore, the prognosis of the disease remains poor. Recent statistics from the US National Cancer Institute showed that the overall 5-year relative survival rate for 2002-2008 was 5.8%, and nearly 90% of all patients were dead in 1

year from diagnosis, with a median survival less than 6 mo[2,3].

Surgery is still the first line treatment for PC, because it provides the only curable option. Other adjuvant treatments are chemotherapy, radiotherapy, physiotherapy and biotherapy. However, PC is highly resistant to the currently available chemotherapy and radiotherapy, and it is one of the cancers for which survival rate has not been substantially improved during the past 30 years. Therefore, new effective modalities for the treatment of this disease are urgently required. In recent years, with the outstanding progress of modern molecular biology, tumor immunology and gene engineering technology, tumor biotherapy is becoming a perspective and rapidly developing field of modern medicine, which is expected to improve state of or even cure patients who are not curable by classical methods of therapy.

Generally, tumor biotherapy includes immunotherapy and gene therapy, but there is no explicit boundary between them. Gene therapy can be used to transfer genes into tumor cells to render them more highly immunogenic, while cancer immunotherapy utilizes gene engineering technology to produce immunomodulating agents, such as tumor vaccines.

Since the first gene therapy clinical trial was approved by the National Institutes of Health in May 1990, significant progress in gene therapy technology has been achieved. In September 2006, a successful immunogene therapy of two patients with metastatic melanoma was reported. Up to July 2013, we have entries for 1970 trials undertaken in 31 countries, and most of them had been aimed at the treatment of cancer (64.2% of all gene therapy trials)[4]. In 2008, detailed, global, genomic analyses found that PC contained an average of 63 genetic alterations, the majority of which were point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes that were each genetically altered in 67%-100% of the tumors[5]. PC gene therapy is, therefore, targeting genes involved in these cellular pathways, inducing direct cell apoptosis and/or stimulating host immune defense system against tumor growth and expansion. Highly efficient gene therapy regimen is based on the following key points: efficiency of gene delivery, tumor targeted therapy and selection of efficient targets.

STRATEGIES FOR GENE THERAPY

Gene replacement

This strategy seeks chances of replacing a mutated gene with a normal gene *via in situ* homologous recombination. It is the best way to treat or even cure monogenic diseases, but seldom used for cancer gene therapy because of technical limitations and complex genetic alterations in cancer.

Gene modification

This strategy tries to directly modify the mutated gene and rehabilitate functions of target cells. It is an ideal manner of gene therapy but with great difficulties. Rare research related to this strategy has been reported.

Gene augmentation

Gene augmentation intends to transfer exogenous therapeutic genes into deficient cells and let their expression products make up for the deficiency. This is the most commonly used strategy in gene therapy. Key point of this technology is the selection of therapeutic genes and gene delivery systems. Plenty of efficient delivery systems have been developed to introduce genetic material into eukaryotic cells and get them expressed. The details will be discussed below.

Gene blockade

This strategy seeks to prevent the transcription and translation of certain cancer-associated genes by using short nucleotide sequences that bind in a complementary fashion to specific DNA or RNA, which can block aberrant signal transduction pathway and induce tumor differentiation and apoptosis eventually. It is also known as antisense gene therapy. Common materials used in this strategy include antisense oligonucleotides, ribozymes and small interfering RNAs (siRNAs).

Antisense oligonucleotides: Antisense oligonucleotides are short single-stranded segments of DNA or RNA artificially synthesized *in vitro*, which can selectively inhibit the transcription and translation of the target gene through the Watson-Crick base pairing between the antisense nucleotide and the target RNA or DNA. Since DNA is more easily synthesized and stable in body fluid, antisense oligodeoxyribonucleotides have become the most common material used in practice. Various chemical modifications to its backbone have been used to improve oligonucleotide stability, targeting and transduction efficiency[6].

Ribozymes: Ribozymes are RNA molecules with catalytic activity that are capable of sequence specific cleaving of mRNA molecules. They can selectively bind to target mRNAs through the Watson-Crick base pairing and form a duplex, which includes a highly distorted conformation that is easily hydrolyzed. The hydrolysis of the mRNA can be used for targeted suppression of specific genes[6].

However, a shortcoming of ribozymes is that their RNA backbone makes them easy targets for degradation by the ubiquitous RNAases, so these molecules are biologically unstable *in vivo*. Then researchers found another category of ribozymes, which are called DNAzymes or deoxyribozymes. They are analogs of ribozymes with greater biological stability, employing DNA motifs to replace the RNA backbone. Moreover, these DNAzymes are also easy to modify synthetically, thereby generating even stronger, resilient second-generation analogs, which makes them powerful tools for gene suppression applications[6].

siRNAs: siRNAs are short double-stranded RNA seg-

ments with typically 21- to 23-nucleotide bases that are complementary to the target mRNA sequence. siRNAs can be artificially synthesized *in vitro* and directly transferred into target cells, or be produced in the genetically modified target cells, in which a gene encoding siRNA is introduced *via* appropriate vectors, with the help of endogenous RNAase. When entering into the target cell, siRNAs bind to ribozyme compounds and form RNA-induced silencing complexes (RISCs), which bind to the target mRNA and stimulate mRNA degradation mechanisms, such as nuclease activity, that lead to silencing of the particular gene. Compared with other gene blockade technologies, siRNAs are remarkably superior because of their high degree of specificity to mRNAs, nonimmunogenic nature and high resistance to ribonucleases. Since siRNAs do not integrate into the genome, they offer greater safety than plasmid molecules. Furthermore, siRNAs do not have to transfer through the nuclear membrane and therefore require less sophisticated delivery systems, promising faster development and higher efficiencies[6]. Thanks to these advantages, RNA interfering technique has become one of the hotspots in research of gene therapy.

METHODS FOR GENE DELIVERY

Ex vivo delivery

In this system, the recipient cells which are previously explanted from the target tissue or bone marrow are cultured or proliferated *in vitro* and subsequently reinfused into the patient after therapeutic gene transfer. Obviously, only transplantable cells, such as lymphocytes and medullary cells, are acceptable in this method. In cancer therapy, tumor cells can also be cultured and engineered *in vitro*, but usually they are used to secrete cytokines or act as a vaccine. To improve the therapeutic efficacy, positively transfected cells are screened from the total cells for implantation, which gives *ex vivo* delivery higher transduction efficiency than *in vivo* delivery. However, the shortcomings of *ex vivo* delivery are complex operational process and a low survival rate of reimplanted cells[7,8].

In vivo delivery

In this system, gene vectors carrying therapeutic genes are directly delivered into the target tissues or organs, *via* systemic injection, *in situ* injection, oral agents or spray, of which *in situ* injection into local tumor tissue mediated by imaging methods is the most commonly used and ripest technology. Almost all the clinical trials on *in vivo* cancer gene therapy are based on this method, which includes intratumoral injection mediated by CT or ultrasound, tumor main vascular perfusion and gene-eluting stent implantation.

In vivo delivery is superior for its simple operation, easy preparation, independence on cell culture systems and wide range of application, whereas low efficiency of transduction, short curative effect, poor target cell specificity and immunologic problems are the main problems

of this system. *In vivo* delivery might be the most useful strategy in clinical application. If only we overcome the shortcomings of this technique, gene therapy can truly be widespread applied in clinical treatment[9].

VECTOR SYSTEMS FOR GENE DELIVERY

The core problem on whether we choose *in vivo* delivery or *ex vivo* delivery is how to achieve specific gene transfection and highly efficient gene expression in recipient cells. As a consequence, establishing an efficient, safe and specialized delivery system has become the foundation of gene therapy. An ideal gene delivery system should have these characters: (1) non-invasive mode of administration; (2) tumor-specific targeting, including primary lesion and distant metastatic lesion, especially site specific lesion, such as the central nervous system and testis; (3) sustained gene expression; and (4) high insertion capacity, bio-safety, stability and easy preparation.

These vector systems can be divided into two categories: non-viral and viral vector systems. Both of them have been investigated and each of them presents distinct advantages and weaknesses. Viral methods normally offer higher transduction efficiency and long-term gene expression, but it may be associated with toxicity, immunogenicity, mutagenicity, inability to transfer large size genes and high costs. Non-viral methods provide advantages including relative safety, ability to transfer large size genes, less toxicity and easy preparation; they can also be modified with ligands for tissue or cell specific targeting. However, non-viral methods show limitations of low transfection efficiency and poor transgene expression[10,11].

Non-viral vector systems

Non-viral vectors consist of chemical vectors, biological vectors and physical methods of gene transfer to introduce naked DNA (in the form of plasmid DNA), RNA molecules, or oligonucleotides into recipient cells.

Physical delivery

Physical delivery mainly includes microinjection, microparticle bombardment and electroporation.

Microinjection involves the utilization of a micropipette to inject nucleic acid directly into a single living cell at a microscopic level. It is highly efficient since one cell at a time is targeted for DNA transfer. However, this precision is achieved at the expense of time. Microparticle bombardment, also known as ballistic DNA injection, gene gun technology or DNA-coated particle bombardment, is used to transfer plasmid DNA coated with heavy metals, usually gold, tungsten or silver, which are used as payload. These particles can be accelerated by pressurized gas and fire at the target cells or tissues without injuring them. Nevertheless, since direct exposure of target tissues is required, its application is restricted in internal organs. In addition, low efficiency of transfection into the nucleus and plasmid DNA integration into host genome are also problems to be solved. Electroporation

uses high-voltage electrical current to generate transient disruption on the membrane of target cells, which allows the entry of plasmid DNA by diffusion. This technique results in high cell mortality and therefore is not suitable for clinical use.

In conclusion, though significant transfection efficiencies have been achieved using physical techniques, they are extremely difficult to standardize in a clinical setting and are considered laborious, impractical, and invasive[11].

Chemical vectors

Commonly used chemical vectors can be classified into two major types based on the nature of the synthetic material, including cationic lipids and cationic polymers. In recent years, chemical vectors have been widely studied due to their advantages, including safety, large size gene transfer ability, less toxicity, low cost and easiness in preparation.

Cationic lipids (liposomes) are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer[6]. DNA is bound by cationic lipids as a result of electrostatic interaction, which allows for fusion of the liposome with the target cell membrane, endocytosis, and delivery of the DNA into the cytoplasm. Cationic lipids mediated gene transfer is the most promising method in non-viral delivery systems. Although this approach has already been applied in clinical trials, some problems still need to be solved for its better application, including the toxicity and lower transfection efficiency *in vivo*[11].

Cationic polymer is an umbrella term of a wide range of chemical compounds, including: (1) natural polymers such as chitosan; (2) dendrimers such as polyamidoamine (PAMAM); (3) polypeptides such as poly-L-lysine (PLL), polyarginine, polyornithine, histones and protamines; and (4) other polymers such as polyethylenimine (PEI) and polyphosphoester[11]. Their transfection activity and toxicity vary dramatically. When mixed with negatively-charged DNA, positive charges of the polymers allow the formation of polymer/DNA complexes (polyplexes) through electrostatic interaction. Polyplexes are nano-sized transfection units that normally have higher stability than lipoplexes. The contributions of cationic polymers are enhancing the DNA uptake *via* endocytosis, protecting DNA from nuclease degradation and facilitating DNA escape from endosomes. Finally, DNA is released into the cytoplasm and migrates into the nucleus in which transgene expression takes place.

Recently, a combination of cationic polymers with liposomes, called polymer/lipid hybrid system, has also been developed and showed some superiority. To prepare this 3-part (lipid/polymer/DNA) system, DNA is pre-condensed by cationic polymers, followed by the subsequent complexation with liposomes. Using cationic lipids, the polymer/DNA complexes can be further condensed and protected, which can facilitate endocytosis and increase circulating half-life *in vivo*[11].

Furthermore, to improve the tissue or cell specificity of chemical vectors, they are also manipulated with the supplement of ligands or fusogenic peptides, such as transferrin, lectin and epidermal growth factor, which can bind to the receptors on the surface of target cells specifically. This approach is also known as receptor-directed gene transfer.

Biological vectors

Bacteria can be used as gene therapy vectors. When engineered to express the therapeutic transgene, bacteria can introduce both the therapeutic gene and protein product to recipient cells. The types of bacteria used include attenuated strains of *Salmonella*, *Shigella*, *Listeria*, and *Yersinia*, as well as non-pathogenic *Escherichia coli*. For some of these vectors, the mechanism of DNA transfer from the bacteria to the mammalian cell is not yet fully understood, but their potential to deliver therapeutic molecules has been demonstrated *in vitro* and *in vivo* in experimental models[12]. A bacterial cancer vaccine for pancreatic cancer - a live attenuated Listeria strain expressing mesothelin - has entered early-phase clinical trial and demonstrated antitumor effects[13].

In addition, many mammalian cell types can be used as carriers of gene therapy vectors, such as hematological cells and mesenchymal stem cells (MSCs)[14]. MSCs possess natural tropism towards tumors, making them a vehicle for targeted delivery of therapeutic genes into tumors. Many experiments have identified their significant antitumor effects *in vitro* and *in vivo*. However, to effectively use this therapeutic strategy in clinic, we still have to solve a number of technical problems[15].

Viral vector systems

Viral vectors: Viral vectors are the most commonly studied and applied gene delivery systems. More than two-thirds of clinical trials of gene therapy reported are viral therapies. These viruses can use their innate mechanism of infection to enter the cell and transfer DNA molecules into cells without any physical or chemical processing. The therapeutic gene then enters the nucleus, integrates into the host gene pool, and is eventually expressed.

The most common viral vectors in cancer gene therapy are adenovirus (AdV), retrovirus (RV), adeno-associated virus (AAV), lentivirus, herpes simplex virus (HSV), influenza virus, Newcastle disease virus, pox virus, and Epstein-Barr virus (EBV). Both advantages and disadvantages should be considered when selecting a viral vector, including insertion capacity, host range of infection, state of integration into host genome, efficiency of transfection and expression, immunogenicity, bio-safety and difficulty of preparation. The comparison of common viral vectors is shown in Table 1[6,9,16-18].

Oncolytic virus: Referring to viral treatment, there is another related field called oncolytic virotherapy in tumor-targeted gene therapy, which is an emerging treatment modality that uses replication-selective virus (or conditionally replicating virus) to destroy cancers. These natural viruses are genetically modified to be non-pathogenic

Table 1 Comparison of characters of common viral vectors

Virus	Viral genome	Insertion capacity	Host range of infection	State of integration into host genome	Efficiency of gene transfection and expression	Immunogenicity	Titers of preparation *in vitro* (PFU/mL)	Bio-safety
AdV	Double-stranded DNA	38 kb	Broad spectrum (both dividing and non-dividing cells)	No integration	High	High	10^{11}-10^{12}	Safe
AAV	Single-stranded DNA	4.9 kb	Broad spectrum	Site-specific integration on chromosome 19q13.3	High	Low	10^{12}, dependent on helper virus	Safe
RV	Single-stranded RNA	8 kb	Dividing cells only	Integrate randomly	Low	Low	10^{6}-10^{7}	Risk of insertional mutagenesis
Lentivirus	Single-stranded RNA	8 kb	Broad spectrum	Integrate randomly	High	Low	10^{9}-10^{10}	Risk of viral infection and insertional mutagenesis
Pox virus	Souble-stranded DNA	25 kb	Broad spectrum	No integration	High	High, function as immunologic adjuvant	10^{6}-10^{7}	Safe
HSV	Double-stranded DNA	15-30 kb	Nerve cells and epithelial cells, especially neuro- tropic speciality	No integration	High	Moderate	10^{11}-10^{12}	Risk of viral infection

but selectively infectious and cytotoxic to cancer cells. Viruses hijack the host cell's protein factory, disabling its production in favor of viral products, which are intrinsically cytotoxic. The infected host cell eventually lyses, releasing new virions capable of infecting other cells in a "bystander" effect, amplifying and propagating the initial effect of infection. These viruses can also evoke an immune response in the host, but in a tolerable morbidity. In conclusion, two main characteristics of oncolytic viruses are: (1) they replicate selectively in cancer cells and have self-amplification properties; and (2) they have cancer-cell-specific toxicity.

Meanwhile, these oncolytic viruses can also be engineered to carry exogenous genetic materials to produce therapeutic effects such as secreting cytokines and enhancing antitumor immune responses prior to eventual cytolysis[19,20].

Nowadays, oncolytic virotherapy has shown its great potential in cancer therapy, and dozens of clinical trials are under way, some of which have been in phase Ⅲ. The rate of publication of manuscripts on oncolytic virotherapy has now surpassed that on viral cancer gene therapy[20]. In 2005, the Chinese State Food and Drug Administration approved the world's first oncolytic virus for treatment of cancer, an engineered human adenovirus (Oncorine; Shanghai Sunway Biotech, Shanghai, China) for treatment of head and neck carcinoma[21].

Target genes and efficacy

At present, cancer gene therapies are mainly based on two principles: gene augmentation and gene blockade. The former is introducing exogenous genetic materials (therapeutic genes) into cancer cells and let their expression products play a therapeutic role to prevent or reverse the growth of cancer cells, while the latter is inhibiting the excessive expression of intrinsic genes (target genes) in cancer cells. Therefore, the investigated genes of interest can be divided into same categories: therapeutic genes utilized for gene augmentation and target genes for gene blockade (Table 2). Since PC is one of the malignant diseases that have the most complicated genetics and pathogenesis, gene therapy of PC has encompassed almost all these categories mentioned above.

Tumor suppressor genes

p16^{INK4a}, p21$^{CIP1/WAF1}$, p14^{ARF}, retinoblastoma protein (pRb) and p53: The *pRb* gene is a part of gene family that includes two other members, *p107* and *p130*, which collectively repress genes that regulates the G1 to S checkpoint of the cell cycle. Rb family proteins interact with transcription factor E2Fs, which induce the expression of genes needed for DNA synthesis. When bound at E2F-responsive promoters, Rb family proteins help to repress gene expression[22]. Rb proteins can be phosphorylated by cyclin dependent kinases 2, 4 and 6 (CDK2, 4 and 6), resulting in the release of E2F and gene expression[23].

The *p16*^{INK4a} gene (*p16*), located on chromosome 9p21, is deleted in 85% of pancreatic adenocarcinomas[24]. It is the first member to be identified in the INK4 family of CDK inhibitors. The *p16* gene product is tight-binding and inhibitory protein for CDK4 to induce G1 arrest of the cell cycle[25], while *p21*$^{CIP1/WAF1}$ (*p21*) gene product acts as a downstream effector of p53 and mediates G1 cell cycle arrest by inhibiting CDK2. Loss of p21 activity has been observed in approximately 30%-60% of PC specimens[26].

The *p14*^{ARF} (*p14*) gene, also located on chromosome 9p21, shares an exon with *p16*^{INK4a} in different reading frames[27]. *Mdm2* (murine double minute) is a *p53*-inducible gene that normally acts to terminate the *p53*

Table 2 Target genes of gene therapy in pancreatic cancer

Strategy	Categories	Examples
Gene augumentation	Tumor suppressor genes	$p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p14^{ARF}$, Retinoblastoma Protein (pRb), *p53*, *p84 /Thoc1*, *p73*, *Smad4/DPC4*
	Drug sensitivity genes/ suicide genes	Herpes Simplex Virus Thymidine Kinase (*HSV-TK*), Cytosine Deaminase (CD), Nitroreductase (NTR), Cytochrome P450 (CYP)
	Anti-angiogenesis genes	Soluble VEGFR, Soluble FGFR, Endostatin, Thrombospondin-1, Angiostatin, Vasostatin, NK4, Matrix metalloproteinases inhibitors (MMIPs/TIMPs), Somatostatin receptors (SSTR)
	Immune related genes	MHC molecules, Co-stimulatory molecules (B7 family, ICAM-1, LFA-3), Inflammatory cytokines (IL-2, IL-12, GM-CSF, IFN-α, IFN-β, IFN-γ, TNF-α), Tumor antigen (CEA, MUC-1, *etc.*)
	Apoptosis related genes	TRAIL
Gene blockade/ antisense therapy	Oncogenes	K-ras, LSM1/CaSm, HER-2/EerB-2
	MDR	MDR1, MRP family, BCRP
	Proliferation related genes	VEGF, hTERT, COX-2

MDR: Multidrug resistance.
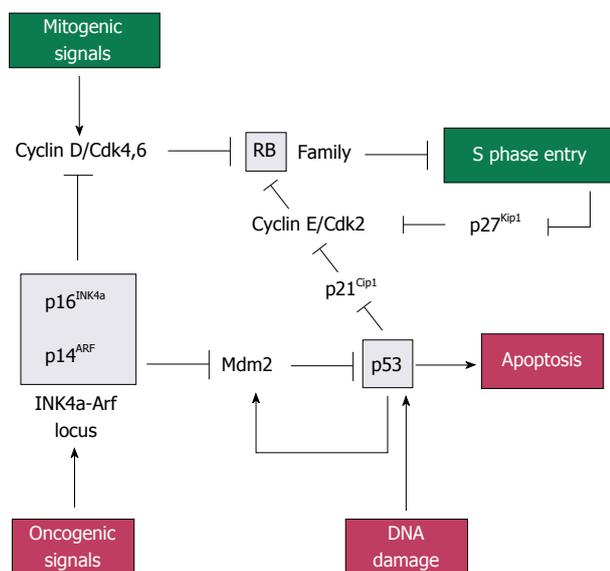
Figure 1 Cell cycle checkpoints. Mitogenic signals activate cyclin D-dependent kinases, which phosphorylate RB and RB family proteins (p107 and p130) to facilitate entry into S phase. The p16INK4a protein inhibits cyclin D/Cdk4, 6 to activate RB and prevent entry into S phase. The p14ARF protein inhibits Mdm2 to induce p53, leading either to p53-dependent apoptosis or to induction of the Cdk2 inhibitor p21Cip1. The p21Cip1 protein inhibits cyclin E/Cdk2 and induces RB-dependent cell cycle arrest.

response. The p14ARF protein inhibits *Mdm2* to induce *p53*, leading to p53-dependent apoptosis[22].

The *p53* gene, located on chromosome 17p, is inactivated by mutation in 70% of pancreatic adenocarcinomas. It can induce apoptosis or G1 cell cycle arrest *via* p21$^{CIP1/WAF1}$. It is normally maintained at a very low level by Mdm2, which targets p53 for ubiquitin-mediated degradation. Stress or mitogenic signals increase the level of p14^{ARF}, which in turn inhibits Mdm2 and lead to the stabilisation and activation of p53.

In short, pRb, p53, p16^{INK4a}, p21$^{CIP1/WAF1}$ and p14^{ARF} form part of a signaling network that monitors mitogenic signaling and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately (Figure 1)[22].

In research of PC therapy, both p16 and p21 have successfully been transduced into pancreatic cancer cell lines by means of adenoviral vectors, which results in growth inhibition and induction of apoptosis *in vitro*[25,28]. One study proved that p16-mediated cytotoxicity is tightly associated with the presence of functional pRb[29]. Liposome-mediated delivery of the p14^{ARF} gene to pancreatic cancer cell lines was capable of resulting in the enhancement of their sensitivity to 5-Fu contrasting with cells devoid of p14^{ARF} expression, which successfully inhibits PC cell proliferation[30].

Of all these tumor suppressor genes, *p53* is the most important and extensively studied one. The first gene therapy for the treatment of cancer in China in 2004 is a replication-defective adenovirus 5 expressing *p53* used for squamous cell carcinoma of the head and neck[21]. Transfer of the *p53* gene using an adenoviral vector also suppressed the growth of human pancreatic cancer cell lines *in vitro*[31]. Reintroduction of *p53* also increased cytotoxicity of gemcitabine *in vitro* and *in vivo*[32], and that of temozolomide *in vitro*[33]. In nude mouse model, intraperitoneal administration of the retroviral *p53* vector resulted in significant inhibition of the growth of primary pancreatic tumor and peritoneal deposits compared to controls[34]. What is more, the inhibitors of Mdm2 are also investigated as new agents for PC treatment, since they induced the growth inhibition through reactivation of the p53 pathway[35].

p84 /Thoc1: The *p84/Thoc1* gene encodes proteins that have similar death domains with other proteins involved in the regulation of apoptosis, which can bind to an amino terminal domain of the Rb1 protein, regulating transcriptional elongation and RNA processing. Its overexpression induces apoptosis of cancer cells. A recent study found that infection of pancreatic adenocarcinoma with adenovirus encoding *p53* and *p84/Thoc1* inhibited growth of cancer cells both *in vitro* and *in vivo* to a greater extent than treatment with either one alone[36].

p73: The *p73* gene, located on chromosome 1p36, is identified as a *p53* family member observed in 45.6% of pancreatic adenocarcinomas. It can induce cell cycle arrest and apoptosis in a p53 manner by binding to *p53* DNA target sites, and transactivates p53-responsive

genes[37]. One research found that an adenoviral vector encoding *p73* was capable of effectively killing several pancreatic cancer cell lines, including those that were completely resistant to p53-mediated apoptosis[38].

Smad4/DPC4: The *Smad4* gene, designated as tumor suppressor gene DPC4, is located on chromosome 18q21.1 and deleted in about 50% of pancreatic adenocarcinomas but only in about 10% or less of other cancers, which suggests that Smad4/ DPC4 may have a specific role in pancreatic tumorigenesis[39]. Smad4 is a member of the Smad family of transcription factors, which potentiates tumor growth, angiogenesis and invasion and is associated with poor prognosis[23]. Restoration of the *Smad4* gene using an adenoviral vector showed inhibition of pancreatic tumor growth in mice[40], while the same effect was seen *in vitro* through a retroviral vector pLXSN containing DPC4[41].

Drug sensitivity genes/suicide genes

Drug sensitivity gene therapy, also known as gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy, attempts to selectively transduce tumor cells with a gene which, when express an enzyme, will convert a systemically administered nontoxic prodrug into a toxic metabolite.

A large number of enzyme-prodrug systems have been developed for suicide gene therapy in recent years. Examples of enzymes include viral thymidine kinase (TK), bacterial cytosine deaminase (CD), bacterial carboxypeptidase G2 (CPG2), purine nucleotide phosphorylase (PNP), thymidine phosphorylase (TP), nitroreductase (NR), D-amino-acid oxidase (DAAO), xanthine-guanine phosphoribosyl transferase (XGPRT), penicillin-G amidase (PGA), β-lactamase (β-L), multiple-drug activation enzyme (MDAE), β-galactosidase (β-Gal), horseradish peroxidase (HRP), deoxyribonucleotide kinase (DRNK), deoxycytidine kinase (dCK), carboxypeptidase A (CPA), β-glucuronidase (β-Glu), and cytochrome P450 (CYP)[42]. However, among these dazzling choices, the most classic paradigm in PC therapy is herpes simplex virus thymidine kinase (HSV-TK).

HSV-TK/ganciclovir

The *HSV-TK* gene codes for an enzyme that converts the nontoxic prodrug ganciclovir into monophosphorylated ganciclovir, which is subsequently further converted by cellular guanylate kinases to the triphosphorylated forms, blocking DNA synthesis and inducing cell death[43]. The therapeutic effect can also be amplified by a "bystander effect", which means *HSV-TK* transduced tumor cells are toxic to neighbouring unmodified tumor cells. The reason may be related to the uptake of toxic metabolites *via* intercellular communication paths such as gap junctions[44]. It is, in some extent, compensable for the low efficacy of gene transfer.

The HSK-TK delivered by retrovirus and adenovirus has been proved efficient in killing PC cells *in vitro* and *in* *vivo*[45,46]. The combination of adenovirus- and retrovirus-mediated delivery of HSV-TK appeared to be more effective in tumor reduction compared to either one alone *in vivo*[47]. Liposome mediated transfer of HSV-TK was able to cause regression of tumors in nude mice with peritoneal dissemination of PC[48]. However, there were some studies showing that retrovirally transduced HSV-TK had limited efficacy in PC cell lines both *in vitro* and *in vivo*[49,50]. The main reason of this controversial fact may be related to a poor efficiency of gene transfection *in vivo* and a limited bystander cell killing effect, so further study is required for its clinical application.

CD/5-fluorocytosine (5-FC)

CD is a bacterial enzyme that converts the prodrug 5-FC into the cytotoxic and radiosensitising agent 5-FU, which inhibits DNA replication and protein synthesis. Several studies have proved that the adenovirus carrying CD gene is efficient in inhibiting the growth of murine PC cell lines *in vitro* and *in vivo* when associated with 5-FC[51-53]. Furthermore, when combined with radiation, the adenoviral vector carrying a mutant bacterial *CD* gene (Ad-bCD-D314A) plus 5-FC significantly increased frequency of tumor regression and the persistence of tumor growth inhibition compared with either radiation or AdbCD-D314A/5-FC therapy alone[54].

There is another gene called *FUR1*, which encodes uracil phosphoribosyltransferase (UPRT), playing a role in CD/5-FC suicide therapy. Since the CD gene, also called *FCY1* gene, often demonstrates resistant to 5-FU, UPRT has an additional advantage as it catalyses the conversion of 5-FU into the toxic metabolite 5-fluorouridine-5'-monophosphate[55]. Combined treatment with 5-FU and E1B-55kDa-deleted adenovirus carrying the *UPRT* gene (AxE1AdB-*UPRT*) dramatically reduced the disseminated tumor burden in mice with peritoneal dissemination of AsPC-1 without causing toxicity in normal tissues[56]. One study showed that the *FCY1* gene alone was ineffective in the treatment of PC *in vitro* and plasmid vectors expressing chimera CD-UPRT (pRSV-CD-*UPRT*) only increased 5-FC sensitivity to some PC cell lines[57]. Another study demonstrated that adenoviral vectors carrying the CD: uracil phosphoribosyltransferase fusion gene (Ad-CD: *UPRT*) resulted in increased 5-FC-mediated cell killing, compared with Ad-CD[58]. Moreover, Ad-CD: UPRT/5-FC combined with monoclonal antibody TRA-8 produces an additive cytotoxic effect in cancer cells both *in vitro* and *in vivo*[58].

Nitroreductase/CB1954

The *Escherichia coli* enzyme nitroreductase (NTR) is able to convert the prodrug CB1954 (5-[aziridin-1-yl]- 2,4-dinitrobenzamide) to 2- and 4-hydroxylamino derivatives, which react with cellular thioesters to generate a potent alkylating agent capable of cross-linking DNA, inducing cell apoptosis eventually[59]. Retrovirus-mediated *NTR* gene delivery showed increased sensitivity up to 500-fold to CB1954 in PC cell lines *in vitro*, through associated by-

Table 3 Active and negative regulatory factors of angiogenesis

Stimulators	Inhibitors
Hypoxia	Angiostatin
Oncogenic proteins such as Ras	TSP-1
Inflammatory cytokines such as IL-8 and IL-6	Endostatin
FGF	Arrestin
TGF-β	Canstatin
HGF	MMPIs
PDGF	Somatostatin
G-CSF	Tumstatin
Angiogenin	VEGI
Leptin	Decoy receptors such as soluble VEGFR
Proliferin	Inflammatory cytokines such as IL-12

G-CSF: Granulocyte colony-stimulating factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; TGF-β: Transforming growth factor-β; FGF: Fibroblast growth factor; TSP-1: Thrombospondin-1; MMPIs: Matrix metalloproteinases inhibitors; VEGI: Vascular endothelial growth inhibitor.

stander effect[60]. In a nude mouse model with subcutaneous PC xenografts, retrovirus vectors expressing the *NTR* gene with administration of CB1954 resulted in tumor regression, growth delay and significantly increased median survival[61]. Another research of nude mouse xenograft model for disseminated peritoneal carcinomatosis with ascites (PC cell line SUIT2) showed that combination of replication-defective adenovirus vectors carrying the *NTR* gene (Ad-CMV-NTR) and *CB1954* almost doubled the median survival from 14 to 26 d[62]. The *NTR/CB1954* treatment has been tested in clinical trials of gastrointestinal and liver malignancies, but none for PC.

Cytochrome P450/cyclophosphamide

Cytochrome P450 enzyme converts the chemotherapeutic prodrugs cyclophosphamide (CPA) or iphosphamide (IPA) to toxic metabolites phosphoramide mustard, which is an alkylating agent able to form DNA crosslinks in a cell cycle-independent manner. Since cytochrome P450 is predominantly produced in the liver and toxicity of the metabolite is rather systemic and not tumor-specific in human body, the transduction of the CYP gene into the tumor tissue shows its advantages to enhance tumor-specific toxicity.

In vitro study demonstrated that expression of CYP 2B1 enzymes (retrovirus-mediated transduction) led to an up to 13-fold increase in susceptibility to IPA in a range of PC cell lines (BxPC-3, MIA PaCa-2, Hs-766T, PaCa-44 and PANC-1)[63]. *In vivo*, retroviral CYP 2B1 transfer with CPA treatment highly sensitized PC cells NP-9, NP-18, and NP-31, and led to significant differences in tumor volume at the end of the treatment when compared with CPA alone[64]. Furthermore, in tumor-bearing mice model, intratumoral injection of encapsulated cells, which were genetically modified to express the CYP, was able to cause significant tumor reduction[65,66]. In addition, these encapsulated cells have also been used in phase Ⅰ/Ⅱ trials in patients with PC and have shown

remarkable early success, with median survival doubled and 1-year survival improved by 3-fold[67].

ANTI-ANGIOGENESIS GENES

Tumor growth is dependent on angiogenesis, in which vascular endothelia growth factor (VEGF) plays a leading role. VEGF is a glycoprotein that has a huge impact on endothelial cell survival, mitogenesis, migration, differentiation, and vascular permeability. It is overexpressed in over 90% of PC and is associated with increased microvessel density, tumor progression and poor prognosis[68]. The VEGF receptor (VEGFR), which is a transmembrane receptor tyrosine kinase of the ErbB family, including VEGFR-1 (FMS-like tyrosine kinase-1, flt-1) and VEGFR-2 [fetal liver kinase-1 (flk-1) or kinase insert domain receptor (KDR)], is also overexpressed in the vasculature of tumors that express VEGF[69,70].

Mechanisms that lead to inappropriate activation of the VEGF pathway include receptor overexpression, activating mutations, overexpression of receptor ligands, and loss of their negative regulatory pathways. Both VEGF and VEGFR are, therefore, appealing targets for anti-angiogenesis therapy. Many molecular targeted agents and monoclonal antibody interfering with VEGF signal system have been developed for cancer therapy, such as Bevacizumab (a humanized antibody against VEGF), Sorafenib (a multi-targeted kinase inhibitor), Erlotinib (an inhibitor of EGFR, the only molecular targeted drug approved by the FDA in 2005 for PC), Axitinib (an inhibitor of both VEGFR and related tyrosine kinase receptors), and Aflibercept (a recombinant fusion protein that functions as a soluble decoy receptor and inhibits VEGF). However, these agents seem unlikely to confer sufficient benefit in the PC clinical trials and their cost-effectiveness has been questioned[71].

Compared to classic non-gene therapy, gene therapy represents a powerful tool for therapeutic intervention to angiogenesis in terms of specific targeting, cost-effectiveness and safety. In this new approach, the VEGF/VEGFR pathway is still the main hotspot. Stimulators and inhibitors that up-regulate and down-regulate VEGF signal pathways are all possible therapeutic targets in cancer treatment (Table 3)[72,73]. Therefore, strategies for anti-angiogenesis gene therapy can be divided into two categories: (1) delivery of genes encoding endogenous angiogenesis inhibitors or their receptors; and (2) blockage of the excessive angiogenesis genes encoding growth factors or growth factor receptors. The former is based on transfer of exogenous genes whereas the latter seeks to block excessive genes in tumor. Materials involved in these two are totally different.

Soluble VEGFR

Soluble forms of VEGFR-1 and VEGFR-2 are a kind of decoy receptor, which can inhibit VEGF dependent tumor angiogenesis, by binding to VEGF and acting as a dominant negative receptor[74]. Recombinant adenovirus-

es encoding soluble VEGFR-2 (Ad Flk1-Fc) and soluble VEGFR-1 (Ad sflt1) showed significant tumor inhibition when injected intravenously and directly into the tumors, respectively[75,76]. Crosslinked polyplex micelles modified by RGD (Arg-Gly-Asp) peptide ligands, a non-viral vector, carrying plasmid DNA expressing a soluble form of VEGFR-1 (sFlt-1), demonstrated significant inhibition of tumor growth *via* anti-angiogenic effect when systemically injected into pancreatic adenocarcinoma bearing mice[77]. A truncated dominant negative mutant of VEGFR-2, which binds to VEGF and decreases the angiogenic stimulus of VEGF, when delivered by replication-defective retroviruses, could also lead to inhibition of tumor growth in each of three human PC cell lines *in vivo*[78]. Similar results were also found in an *in vitro* study with herpes simplex virus (HSV) amplicon mediated delivery of a hypoxia-inducible soluble VEGFR-2 (sFlk-1)[79]. In an comparative research of the antitumor activity of anti-angiogenic proteins, recombinant adenoviruses encoding angiostatin, endostatin, neuropilin, and soluble forms of VEGFR (Flk1, Flt1) all resulted in inhibition of tumor growth through intravenous injection in murine models involving lung cancer, fibrosarcoma and PC, but soluble forms of VEGFR were significantly more effective (approximately 80% inhibition of preexisting tumor growth) than the others[80].

Soluble fibroblast growth-factor receptors (FGFRs)

FGFRs, encoded by four genes (*FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*), are involved in the regulation of organ development, cell proliferation and migration, angiogenesis and other processes. Recent studies have shown that FGFR-activating mutations and overexpression are closely associated with the development and progression of tumors in human[81]. Several monoclonal antibodies and small-molecule FGFR inhibitors have been developed, some of which have already entered early clinical development, such as AZD4547 (AstraZeneca), BGJ398 (Novartis), LY2874455 (Eli Lilly), GP369 (Aveo) and HuGAL-FR21 (Galaxy). Soluble forms of FGFR (sFGFR) had similar mechanism of action with sVEGFR. One study found that replication-defective adenoviral vectors carrying *sFGFR1* gene could effectively suppress tumor angiogenesis and enhance apoptosis among lung cancer cells and pancreatic cancer cells both *in vitro* and *in vivo*, especially in sVEGFR-resistant cancers. Furthermore, the combined usage of sVEGFR plus sFGFR1 produced an enhanced inhibitory effect compared to their individual effects[82].

Endostatin (ES), thrombospondin-1 (TSP-1), angiostatin (AS) and vasostatin

ES, AS, vasostatin and TSP-1 are the most important endogenous angiogenesis inhibitors that have been studied extensively. ES is a cleavage product from the C-terminal portion of collagen XVIII, which has been shown to inhibit endothelial cell migration and proliferation and induce apoptosis. The apoptotic effect of ES is associated with down-regulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-xl, while inhibition of endothelial cell proliferation and migration is *via* interacting with several pathways, such as binding to integrin $\alpha 5$, associating with heparan sulfate proteoglycans on cell surface, and inhibiting VEGFR-2, PDGF, cyclin-D1 and metalloproteinases[83,84]. Recombinant human ES has been developed for cancer treatment and entered in clinical trials[85,86]. TSP-1 is a multifunctional extracellular matrix protein with pivotal roles in the regulation of vascular development and angiogenesis[87], while AS is a potent inhibitor of angiogenesis, selectively inhibiting endothelial cell proliferation and migration through binding a cell surface ATP synthase or inhibiting extracellular matrix (ECM)-stimulated plasminogen[72]. Vasostatin, the N-terminal domain of calreticulin, was also reported to have tumor suppressor and anti-angiogenic function *in vitro* and *in vivo*.

A double-regulated duplicative adenovirus expressing human ES (AdTPHre-hEndo) limited PC growth both *in vitro* and *in vivo*, and the inhibition was significantly higher than non-duplicative adenovirus vectors carrying the ES gene[88]. A eukaryotic expression vector pRC/CMV carrying the AS gene, when delivered into human PC cell line mediated by liposome *in vitro*, also notedly reduced the volume of tumors[89]. A replication deficient recombinant adenovirus encoding vasostatin (Ad-vasostatin) showed less neovascularisation and inhibited tumor growth *in vivo* and *in vitro*[90]. Same results were also discovered in a study with intratumoral delivery of recombinant AAV expressing vasostatin (rAAV-VAS)[91]. The Lister vaccine strain of vaccinia virus armed with the ES-AS fusion gene (*VVhEA*), which displayed high selectivity for cancer cells with effective infection of tumors after both intravenous and intratumoral administrations, showed a significant antitumor effect with evidence of inhibition of angiogenesis both *in vitro* and *in vivo*[92]. A recombinant AAV-mediated gene delivery of 3TSR (the antiangiogenic domain of TSP-1) or ES (rAAV-ES), *via* intratumoral injection, intramuscular injection or intrasplenic injection, also inhibited tumor growth by anti-angiogenic effect[87].

NK4

NK4, composed of the N-terminal hairpin and subsequent 4-kringle fragment of hepatocyte growth factor (HGF), is an HGF antagonist that acts as an angiogenesis inhibitor. NK4 strongly inhibits the infiltration, metastasis, and tumor growth of PC[93]. HGF is overexpressed in 61%-87% of PC cases[94] but frequently expressed in tumor-associated fibroblasts rather than PC cells. HGF promotes growth and enhances cell motility and extracellular matrix breakdown, leading to invasion and metastasis of cancer cells[95].

Strong antiangiogenic activity of an NK4-expressing adenvirus vector (Ad-NK) has been tested on PC cells in both *in vitro* and *in vivo* studies of subcutaneous and orthotopic transplantation, peritoneal dissemination, and liver metastasis[96]. Ad-NK potently inhibited the growth

and invasion of cancer cells in response to HGF[97]. Intrasplenic injection of Ad-NK4 suppressed the number and growth of hepatic metastases[98]. When injected into the peritumoral region combined with gemcitabine, Ad-NK4 completely suppressed peritoneal dissemination and liver metastases, leading to significantly increased survival, compared to Ad-NK or gemcitabine alone[96]. Intraperitoneal injection of Ad-NK4 could also suppress the development of peritoneal dissemination of PC in nude mice[99].

Matrix metalloproteinase (MMP) inhibitors

MMPs are a family of zinc-dependent proteolytic enzymes that degrade the extracellular matrix and play an important role in tumor progression, angiogenesis, immune evasion, invasion and metastasis[100]. Natural tissue inhibitors of metalloproteinases (TIMPs) regulate physiologic MMP activity and are implicated in malignancy. The imbalance between MMPs and TIMPs, which denotes overexpression of MMPs and reduced expression of TIMPs, is frequently found in PC[101]. Some synthetic MMPIs have been exploited for antitumor therapy, such as BAY 12-9566 and Marimastat, but showed limited survival benefit in clinical trials in spite of their effectiveness in animal models[102-104]. When undergoing gene transfection to overexpress *TIMP-1 in vitro*, human PC cells were less likely to implant, grow and migrate in nude mice and showed increased apoptosis, and decreased angiogenesis[105]. Adenoviral vectors encoding human TIMP1 or TIMP2 limited development of PC and led to prolonged survival *in vivo*[106].

Somatostatin (SST) receptors (SSTRs)

SST is a natural peptide hormone secreted in various parts of the human body, and participates in a wide variety of biological processes including neurotransmission and negative control of exocrine and endocrine secretions. Meanwhile, SST exerts a strong antiproliferative effect in normal as well as tumor cells by interacting with SSTRs, including apoptotic effects, growth factor inhibition, antiangiogenic and immuno-modulating activities[107,108]. SSTRs consist of five different G-protein coupled receptor subtypes (SSTR1-5), which are differently expressed in the various types of tumor. The antineoplastic activity of SST and its analogues depends on the receptor subtypes they are bound to. Among these receptor subtypes, SSTR-1 and SSTR-2 play a predominant role in mediating the anti-proliferative effect[109]. However, only *SSTR2* expression is significantly inactivated in 90% of PC cases and SSTR-2, therefore, occupies the majority of gene therapy studies of SSTRs[110,111].

When introduced into PC cell line PC-3 with lipofectamine, SSTR2 showed inhibition of *VEGF* and *MMP-2* expression *in vitro*[112]. A targeting adenoviral vector driven by MUC1-promoter expressing SSTR2 gene showed significant cell proliferation inhibition *in vitro*, though there was no AdMUC1-SSTR2-induced apoptosis[113]. Intratumoral transfer of SSTR2 using the

synthetic vector linear polymers of ethylenimine (PEI), strongly inhibited tumor progression of pancreatic adenocarcinoma *in vivo*, while depleting SST by RNA interference completely reversed SSTR2's antitumoral effect on *VEGF* expression and tumor angiogenesis[107]. Another study demonstrated that SSTR2 restoration mediated by oncolytic adenovirus (ZD55-hSSTR2) alone had a minor antitumor effect, but antitumor efficacy can be enhanced with the combination of ZD55-TRAIL (TNF-related apoptosis-inducing ligand) *in vitro* and *in vivo*[114]. Gene transfer using SSTR1 also displayed growth inhibition of PC by inducing cell cycle arrest *in vitro* and *in vivo*[115]. What is more, cotransfection of *SSTR1* and *SSTR2* showed a synergistic inhibitory effect on tumor proliferation and rendered Panc-1 cells more responsive to an SST analogue[116].

IMMUNE RELATED GENES

Tumor cells generally have a low immunogenicity and are able to escape surveillance by the host. Cancer immunotherapy has been developed to overcome this immune tolerance, including active and passive immunity. Passive approach encompasses administration of cytokines, activated effector cells or specific monoclonal antibodies targeting tumor cells. Active immunotherapy involves stimulation of immune response to tumor-associated antigens (TAAs), *via* employing cancer vaccines. Meanwhile, gene therapy is helpful by transferring genes into tumor cells or immune cells to render them more immunogenic and more effective, respectively. This combination is also known as immunogene therapy (Figure 2)[117].

In pathway A, allogeneic or autologous tumor cell vaccines that are genetically modified to secrete cytokines or co-stimulatory molecules are designed to elicit systemic immune responses to attack tumor tissue. Though autologous tumor cell vaccines showed promising results in clinical trials, a number of technical problems were uncovered, including the requirement of labor-intensive procedures for production of an individualized vaccine and the difficulty of expanding primary human tumor cells to the high numbers required for vaccination[118]. Fortunately, allogeneic whole tumor cell vaccines had been also proved to successfully induce systemic tumor-specific immune responses without the need to be HLA compatible with the host, and became the major way in study of tumor vaccines[119]. In pathway C, immune cells, especially dendritic cells (DCs), are *ex vitro* cultured autologous cells that are genetically modified, stimulated by specific antigens or activated by multiple cytokines to bypass the dysfunction of endogenous immune cells, restore immune surveillance, induce cancer regression or stabilization or delay and prevent its recurrence.

Host immune responses against a tumor antigen include cellular and/or humoral immune responses, starting with the processing of tumor antigens by antigen presenting cells (APCs) and recognition by T lymphocytes. T-cell activation requires not only the interaction between

A

Cancer cells

Gene

Cells killed

Immune system activation

Tumor cell destruction

B

Gene

Cancerous tissue

Immune system activation

Tumor cell destruction

C

Immune cells

Gene

Live cells

Immune system activation

Tumor cell destruction
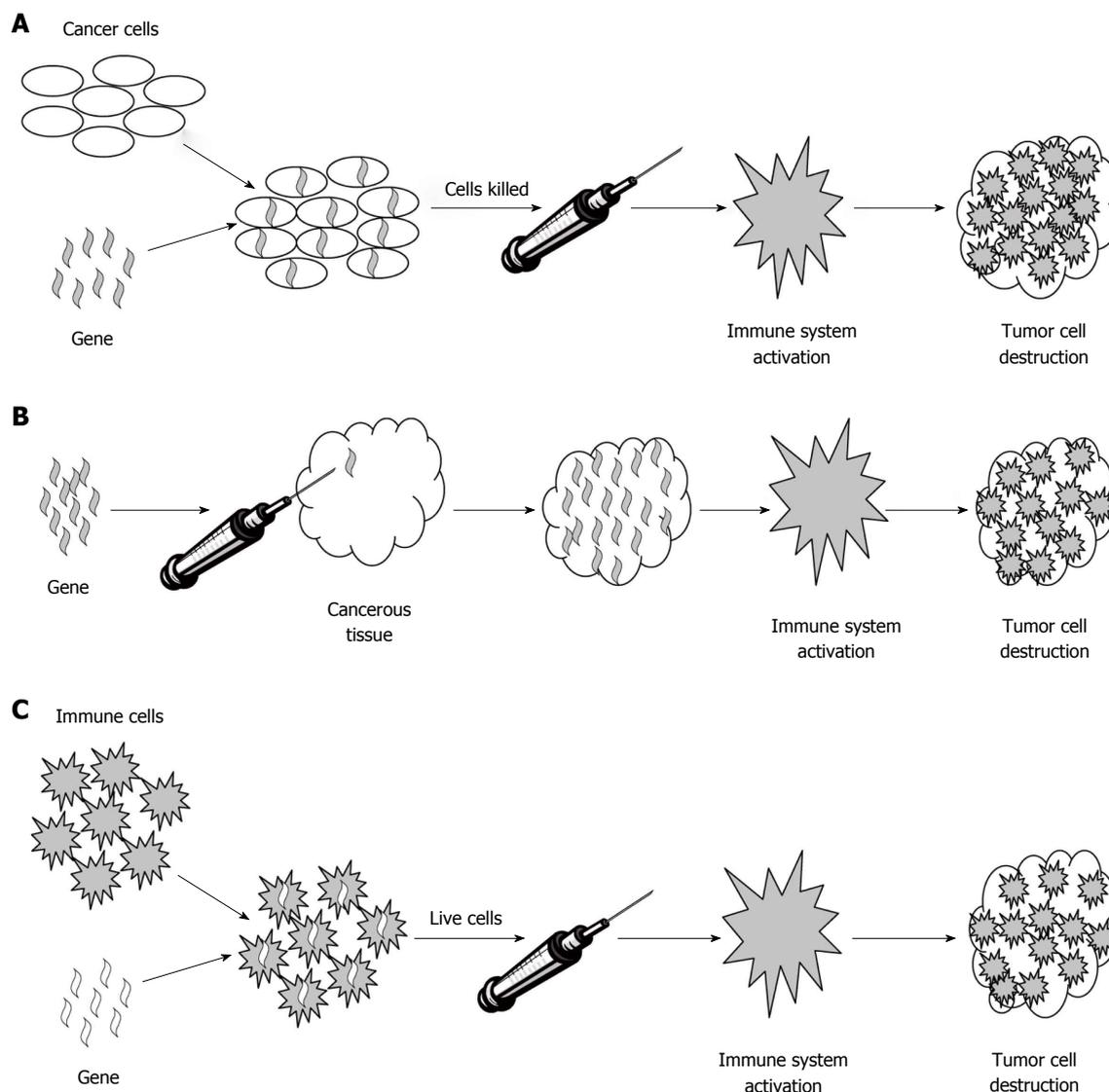
Figure 2 Schematic diagram of immunogene therapy. A: Immunogene therapy with altered cancer cells, which are harvested from patients (autologous cells) or from established cancer cell lines (allogeneic) and then cultured *in vitro* and inactivated to yield a vaccine; B: Immunogene therapy with *in vivo* gene transfer; C: Immunogene therapy using altered immune cells.

major histocompatibility complex (MHC) antigens bearing a specific peptide and the T-cell receptors, but also non-antigen-specific co-stimulatory activation by interaction of molecules expressed on the T-cells and APCs. Mechanisms of tumor immune tolerance include: underexpression of MHC antigens, loss of co-stimulatory molecule expression, alteration of tumor antigens, and secretion of immunosuppressive factors. Aiming at these mechanisms, at least four kinds of genes can be utilized in immunogene therapy: (1) major histocompatibility complex (MHC) antigens/human leukocyte antigens (HLAs), (2) co-stimulatory molecule genes, (3) tumor antigen genes; and (4) inflammatory cytokine genes.

MHC molecules and co-stimulatory molecules

Tumors may be capable of delivering antigen-specific signals to T cells, but may not deliver the co-stimulatory signals necessary for full activation of T cells. Therefore,

one approach to induce tumor-specific immune responses is to genetically modify tumor cells to express MHC molecules and co-stimulatory molecules on their cell surface. This kind of modification makes tumor cells to function as professional APCs and enhance their ability to directly stimulate T cells. In addition, this way is also appropriate for manipulation of DC cancer vaccines[120].

Loss or reduced expression of MHC-class-I molecules in many cancer cells have been found in mice and humans and have been identified as an important form of immune evasion. Despite that a large number of potential cancer antigens were discovered, clinical trials of immunization were disappointing due to the loss of *MHC-I* expression in tumors[121]. In animal models, *in vivo* gene transfer of foreign MHC-class-I *H-2K* gene into tumors successfully induced a cytotoxic T-cell response and attenuated tumor growth and caused complete tumor regression in murine models[122,123]. Transfection of

tumor cells with syngeneic MHC-class-Ⅱ or allogeneic MHC-class-Ⅰ genes improved tumor-specific immunity in the autologous host[124]. Therefore, combining MHC molecules with other tumor antigens could emerge as an attractive approach in cancer immunotherapy. No correlative research in PC has been reported yet.

In studies of costimulatory molecules, B7 family is one of the most important members and is constitutively expressed by most APCs, as the ligand for two receptors expressed on T cells, CD28 and CTLA-4. In animal studies, transfection of B7.1 into some tumors resulted in tumor rejection and generated systemic immunity against wild-type tumor challenges *via* stimulating CD8+ T cells[125]. Cotransfection of B7 with MHC-Ⅱ molecules was capable of inducing potent systemic immunity *via* both CD4+ and CD8+ T cells pathways[126]. A combinatory vaccine regimen (PANVAC-VF) composed of vaccinia virus and fowlpox virus expressing tumor-associated antigens (CEA and MUC1) and costimulatory molecules [B7.1, intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated antigen-3 (LFA-3)], when administered by subcutaneous injection with adjuvant GM-CSF, showed a significantly great therapeutic effect both in animal models and in clinical trials with advanced PC patients[127,128].

Inflammatory cytokines
Numerous cytokines have been studied in *in vivo* gene transfer. Human PC cells that underwent retrovirus-mediated gene transfer of IL-2, IL-4, IL-6, IL-27 and granulocyte macrophage-colony stimulating factor (GM-CSF) showed significant retardation and even regression when inoculated into BALB/c nude mice[129,130]. *In vivo* gene delivery of IL-1β, IL-24, IFN-α, IFN-β and IFN-γ by different viral vectors have been proved to lead to significant tumor growth inhibition in many PC models[131-135]. In addition, combined application of cytokine transfection and traditional chemotherapy, and combining immune genes with either tumor suppressor genes or suicide genes, are also common strategies in cancer immunogen therapy. For instance, combination of intratumoral human TNF-α gene delivery with gemcitabine produced marked delays in the growth of human pancreatic xenograft tumors relative to either agent alone *in vivo*[136,137]. Combination of IFN-α gene delivery with 5-FU had similar results as well[137]. An *in vitro* study of combining INF-β gene tranfection with gemcitabine also showed tumor growth inhibition[138]. In a phase Ⅰ/Ⅱ trial, intratumoral gene delivery of replication-deficient adenovirus encoding TNF-α, combined with standard chemoradiation, showed promising clinical outcome with dose-limiting effect and toxicity[139].

In studies of tumor cell based vaccines, cytokine genes have been also introduced into tumor cells to render them tumorigenicity and immunogenicity. One study that directly compared and contrasted effects of different cytokines in murine tumor models demonstrated that the tumors transduced with GM-CSF produced the greatest degree of systemic immunity relative to irradiated non-transduced tumor cells[140]. In a phase Ⅰ trial of allogeneic GM-CSF-secreting cancer vaccines in 14 patients with pancreatic adenocarcinoma, no local or systemic dose-limiting toxicities were observed and three subjects who received the highest two dose levels showed increased disease-free survival time (more than 2 years)[141]. In a comparative clinical trial of GM-CSF-secreting cancer vaccines alone and combined with cyclophosphamide in patients with metastatic PC, minimal treatment-related toxicity was found and cyclophosphamide cohort exhibited longer progression-free survival and overall survival[142]. Another clinical trial of ipilimumab (anti-CTLA-4) in combination with allogeneic PC cells transfected with a *GM-CSF* gene also showed prolonged disease stabilization in previously treated advanced PC[143].

Tumor antigens
Clinical studies of immunotherapy in cancer have focused on five classes of tumor antigens: (1) tumor-specific antigens (MAGE-1, NY-ESO-1, TRAG-3, PSA); (2) mutated oncogene products (p53, K-ras, HER2, BCR/abl, WT-1); (3) reactivated embryonic gene products (CEA, AFP); (4) self-antigens overexpressed in tumors (MUC1, survivin); and (5) oncogenic virus antigens (EBV, HPV, HBV)[120,144]. Many antigen-specific vaccines that urge the host immune system to recognize the primary tumor have been developed, including recombinant viral and bacterial vaccines that encode tumor antigens, peptide-or protein-based vaccines that mixed with adjuvants, DNA-based vaccines expressing tumor antigens, and antigen-pulsed DC vaccines[120]. Up to now, at least 75 antigens were identified that had many of the study-defined characteristics of an ideal candidate antigen for cancer therapy. Many of these antigens are the focus of targeted therapy in clinical trials in cancer (Table 4)[144-149].

It is encouraging that many of the results of PC vaccine trials verified the safety and immunogenicity of these vaccines. In a mutated K-ras peptide vaccine clinical trial with 48 patients in PC, when combined with adjuvant GM-CSF, more than 50% of patients demonstrated a tumor-specific immune response and significantly improved median overall survival *vs* their non-responding counterparts[150]. A multi-institution double-blinded placebo-controlled trial of gastrin peptide vaccine in 154 patients with advanced-stage PC demonstrated a nearly 2-fold increase in the median overall survival in the treatment compared to the placebo group[151]. A phase Ⅰ/Ⅱ clinical trial of telomerase peptide vaccines also demonstrated prolonged survival in immune responders *vs* non-responders[152]. In a multi-institutional, open-label, dose-finding, phase Ⅱ trial of Algenpantucel-L, 70 patients with resected PC were administered by two different doses of the vaccine in combination with gemcitabine and 5-fluorouracil. Of the PC patients who received a higher dose of vaccine, 96% survived for at least one year in comparison to the historical control of 69%, showing a statistically significant difference[153].

Peptide or protein vaccines	Mutant K-ras peptide
	WT1 peptide
	CAP1-6D
	G17DT
	HLA-A*0201 restricted VEGF receptor-1 and -2 peptides
	HLA-A24 restricted survivin-2B80-88 peptide (AYACNTSTL)
	Autologous heat shock protein HSPPC-96
	TELOVAC
Whole cell vaccines or dendritic cell vaccines	α-Gal transferase transfected allogeneic tumor cells (including Algenpantucel-L)
	GVAX
	MUC-1 pulsed autologous dendritic cells
Viral or bacterial vaccines	PANVAC-VF-MUC-1, CEA, and TRICOM transfected virus
	Mesothelin (CRS-207) transfected live-attenuated Listeria
DNA vaccines	VXMO1

VXMO1: VEGF receptor-2 DNA vaccine; GVAX: GM-CSF transfected allogeneic tumor cells; TELOVAC: Telomerase peptide GV1001; CAP1-6D: CEA peptide; WT1: Wilms tumor 1; G17DT: Gastrin peptide.

Although encouraging, results from single-agent immunotherapy clinical trials have been underwhelming. As more and more tumor antigens are identified, more specific and potent vaccines will be developed. The ideal vaccine will target multiple antigens that are crucial to the growth and progression of tumors. Perhaps combinatorial therapeutic approach, which includes chemotherapy, radiation, surgery, and immunotherapy, will result in even greater survival benefits for patients with PC[13].

APOPTOSIS RELATED GENES

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL or Apo-2 ligand (Apo-2L) is a type II transmembrane protein belonging to the TNF superfamily and serves as an effective anticancer agent due to its cancer cell specificity and potent antitumor activity. TRAIL interacts with death receptor 4 (DR4) and DR5, which form the death-inducing signal complex (DISC) by binding to Fas associated death domain (FADD), thereby activates caspase-8 and results in activation of downstream caspases-3, -6, and -7, and apoptosis induction[154]. Recombinant human TRAIL has been developed as a novel anticancer agent and is being clinically evaluated for the treatment of both solid tumors and hematological malignancies, such as colorectal, melanoma, lung, ovarian cancers and non-Hodgkins lymphoma[155]. However, its use *in vivo* is limited by a short half-life in plasma due to a rapid clearance by the kidney.

Gene delivery of TRAIL into tumors may overcome this limitation. An adenoviral vector expressing TRAIL driven by a human telomerase reverse transcriptase (hTERT) promoter showed specific antitumor efficacy in PC cell lines *in vitro* and significantly suppressed tumor growth *in vivo*[156]. Systemic administration of this vector in combination with chemotherapy (gemcitabine) exhibited a synergistic effect in the induction of apoptosis[157]. TRAIL-engineered pancreas-derived mesenchymal stem cells (MSCs) were able to induce PC cell apoptosis *in vitro*[158]. Another study of adipose-derived MSCs that were transduced with the TRAIL gene showed that, when injected intravenously or subcutaneously into mice, these MSCs localized into tumors and mediated apoptosis without significant apparent toxicities to normal tissues[159]. These studies implied that MSCs may serve as a stable source of TRAIL delivery in PC therapy.

ONCOGENES

K-ras

Ras is the most common oncogene detected in human cancers. It comprises 3 families, H-ras, K-ras, and N-ras. Of these, the K-ras family is responsible for almost all of the PC mutations, with mutations in the other families occurring rarely. Studies suggested that K-ras, which is located on chromosome 12p13, is mutated in up to 95% of PC cases[16,160]. The *K-ras* encodes membrane-bound GTP-binding proteins, which can be activated by signaling partners to regulate many cellular functions, including cell growth, proliferation, and differentiation. Mutations of *K-ras* result in malfunction of GTPase and participate in the initiation or early phase of pancreatic tumorigenesis[71].

To blockade the Ras signaling pathway, cancer vaccines that stimulate immunity against mutant Ras proteins and antisense therapy that blocks the translation of mutant Ras gene are two common strategies. Antisense therapy involves the use of oligonucleotides, ribozymes and siRNAs. Retroviral delivery of K-ras siRNA to human tumor cells induced loss of expression of the K-ras gene, leading to loss of anchorage-independent growth and tumorigenicity *in vitro*[161]. Another study of K-ras siRNA delivered by electroporation demonstrated significant tumor growth inhibition both *in vitro* and *in vivo*. When K-ras siRNA is combined with gemcitabine, survival rate was significantly prolonged and the mean tumor volume was dramatically reduced when compared with single agents[162].

K-ras antisense oligodeoxynucleotide (K-ras-ASODN) was identified to successfully inhibit *K-ras* expression[163] and suppress the growth and invasiveness of PC cell lines *in vitro*[164]. In peritoneal dissemination models of PC, intraperitoneal injection of adenovirus expressing antisense K-ras RNA significantly suppressed the peritoneal growth with no significant systemic toxicity[165]. K-ras ASODN combined with type I insulin-like growth factor receptor (IGF-IR) antisense oligodeoxynucleotide (IGF-IR-ASODN) showed a significant inhibitory effect on tumor growth and induced apoptosis *in vitro* and *in vivo*, compared with each agent alone[166]. In a phase II trial of pa-

tients with locally advanced and metastatic PC, ISIS 2503, a phosphorothioate oligonucleotide antisense inhibitor of human H-ras mRNA, showed a response rate of 10.4% and a median survival of 6.6 mo in combination with gemcitabine, which is promising but of unclear benefit[167]. Initial enthusiasm for this approach is currently diminishing following the failures of antisense inhibitors such as ISIS 3521 (a protein kinase C-alpha antisense oligonucleotide) and oblimersen (a bcl-2 antisense oligonucleotide) in lung cancer and melanoma, respectively.

LSM1

The cancer-associated Sm-like (CaSm) oncogene LSM1 has been reported to be overexpressed in 87% of PC cases[168]. An adenovirus expressing CaSm antisense RNA (Ad-alpha CaSm) reduced endogenous CaSm mRNA expression *in vitro*, and a single intratumoural dose of Ad-alpha CaSm inhibited tumor growth and extended survival time in an *in vivo* SCID mouse model of human PC. The antitumor effect was further enhanced by gemcitabine[168]. In a metastatic tumor model, systemic administration of Ad-alpha CaSm resulted in a significant decrease in the number of hepatic metastases and increased survival time through both direct and bystander effects[169].

HER-2/ErbB-2

Human epidermal growth factor receptor 2 (HER-2) is a transmembrane receptor tyrosine kinase of the ErbB family. It participates in the EGFR signaling pathway and has roles in cell proliferation, survival, motility, invasion and adhesion. Blocking of overexpressed HER-2 oncogene was able to improve survival in breast and gastroesophageal cancers. Some studies found that HER-2 amplification occurs in 2% of pancreatic ductal adenocarcinomas (PDACs) but has distinct features with implications for clinical practice, which represents an attractive target for anti-HER2 therapies[170]. However, clinical trials of anti-HER2 antibodies such as trastuzumab showed no benefits compared with standard chemotherapy and did not recommend further evaluation of anti-HER2 treatment in patients with metastatic PC[171].

MULTIDRUG RESISTANCE GENES

Drug resistance is a major cause of treatment failure in cancer chemotherapy. One of the important mechanisms of tumor multidrug resistance is increased drug efflux and decreased accumulation of drugs in the cell. Efflux transporters of the ATP-binding cassette (ABC) family such as ABCB1 (multidrug resistance 1, MDR1), the ABCC (multidrug resistance-associated protein, MRP) family, and ABCG2 (breast cancer resistance protein, BCRP) have been identified as major determinants of chemoresistance in tumor cells[172].

MDR1 is a classic paradigm in the ABC family and has been analyzed in detail in PC. Its product, P-glycoprotein, is a membrane protein that functions as an ATP-dependent exporter of drugs from cells. Some studies

demonstrated a high rate (73.2%) of *MDR1* expression in PC[173,174], and other studies reported that MDR1 is associated with sensitivity to gemcitabine[175]. The MRP family consists of 9 members (MRP1-9) and is involved in exporting a variety of endogenous substrates as well as organic anions of xenobiotics, conferring cells resistance to cytotoxic and antiviral drugs[176]. It has been shown that the expression of MRP3 and MRP5 mRNAs was upregulated in PC and MRP3 was even correlated with tumor grade[176]. BCRP was first derived from a resistant breast cancer cell line, but is present in a wide range of human solid tumors, including PC[177]. One study proved that BCRP expression was frequent (73.1%) in PC, and high BCRP expression was a significant prognostic factor for early tumor recurrence and poor survival[178]. Conversely, a study demonstrated low BCRP mRNA levels in both normal pancreatic tissues and PC[176].

In fields of anti-*MDR* gene therapy for PC, some studies have showed that siRNAs against *MDR1* could specifically inhibit *MDR1* expression at the mRNA and protein levels and decreased resistance against daunorubicin in PC cell lines *in vitro*[179]. Similar results were found in another study of a hammerhead ribozyme against MDR1 mRNA[180]. However, *in vivo* studies and clinical trials are still scarce in this field and there is much room for advancement to validate their clinical applicability.

Another important approach of making use of the *MDR* gene is transducing the *MDR* gene into hematopoietic stem cells to strengthen the host's resistance to myelosuppression caused by chemotherapeutic drugs. An advantage of this approach is that it permits higher doses of chemotherapy without severe adverse effects, thus providing a better chance to achieve remission. *In vitro*, lentiviral or retroviral vectors encoding MGMT (P140K) and MDR1 or MRP1 resulted in significant survival advantage of human hematopoietic stem cells when undergoing intensification chemotherapy, compared with untransduced cells or either single vector alone[181-183]. In mouse models, *MDR1* gene modified hematopoietic cells also showed chemoprotective effect from various anticancer drugs[184,185]. So far, several clinical trials of MDR1 transfected autologous hematopoietic stem cell transplantation have been approved for the treatment of patients with breast cancer, ovarian cancer or leukemia. However, since the expression rate of the *MDR1* gene in PC is very high, this system may not be useful for treating this cancer.

PROLIFERATION RELATED GENES

VEGF

As mentioned above, the VEGF signal pathway plays a leading role in tumor angiogenesis. Therefore, knockdown of VEGF gene expression is a promising way in anti-angiogenesis therapy in PC. Materials in this approach include antisense oligonucleotides, ribozymes and siRNAs.

One study of antisense oligodeoxynucleotide of

VEGF-C showed that it decreased the expression levels of VEGF-C and inhibited lymphangiogenesis in nude mice with orthotopically xenografted human PC, but had no significant effect on angiogenesis[186]. This result was verified in another study with short hairpin RNA (shRNA) targeting *VEGF-C*, and the VEGF-C shRNA significantly inhibited cell proliferation and tumor growth *in vivo*[187]. On the other hand, antisense oligonucleotide of VEGF was proved to significantly decrease neoangiogenesis and vascular permeability in orthotopic xenograft models; furthermore, it reduced tumor growth and metastasis and improved survival[188]. Systemic or intratumoral injection of VEGF specific siRNAs also led to the significant reduction in the subcutaneous tumor growth through down-regulating VEGF expression and decreasing microvascular density[189,190]. A study with hammerhead ribozymes against VEGF gene transcripts showed inhibition of tumor growth and liver metastasis of a PC cell line *in vivo*[191]. However, no clinical trial in this approach has been reported in PC.

Human telomerase reverse transcriptase

Telomere is a region of repetitive nucleotide sequences at each end of a chromatid, which plays a critical role in maintaining chromosome stability. Telomere is shortened progressively during normal cell division. When its length becomes critically short, it triggers replicative senescence or apoptosis. Telomerase is a ribonucleoprotein polymerase that maintains the length of telomere. Human telomerase complex consists of telomerase reverse transcriptase (hTERT), telomerase RNA (hTR or TERC), telomerase associated protein-1 (TEP-1), hsp90 and p23, of which the RNA subunit and hTERT constitute the core of telomerase. It has been tested that hTERT is the limiting component of telomerase and its expression levels parallel to those of telomerase activity[192,193]. Generally, telomerase activity is detectable only in germ line cells and certain stem cells but is repressed in somatic cells. Upregulated telomerase activity is associated with promotion of tumorigenesis, neoplastic growth and metastasis of human cancer[194,195]. In fact, approximately 85% of human cancers exhibit reactivation of telomerase activity, which is even as high as 92%-95% in PC[196,197]. Recently, one new viewpoint declares that hTERT is also implicated in DNA repair and regulation of the expression of genes that control cell proliferation, which promotes tumorigenesis independent on the stability of telomere[198]. In a nutshell, hTERT is an important proliferation-related factor and can serve as a tumor marker and a prognostic indicator.

In vitro, hTERT antisense oligonucleotide (hTERT-ASODN) could down-regulate expression of hTERT mRNA and increase cell apoptosis rate in a concentration- and time-dependent manner in PC cell lines[199,200]. Therefore, consecutive transfections were performed in order to inhibit telomerase activity and result in a continuous reduction in cell viability[201]. Cell cycle analysis indicated that the cells were mainly arrested at the G0/G1 phase with the treatment of hTERT-ASODN. In addition, hTERT ASODN synergized with gemcitabine to exert an anti-proliferation effect[200]. Similar results were demonstrated in another study of hTERT-siRNA transfection in pancreatic cancer cell line Capan-2, and the inhibitory effect was associated with the downregulation of *Bcl-2* and cyclooxygenase-2 (*COX-2*)[202]. Hammerhead ribozyme targeting hTR was also found to depress telomerase activity, and ribozyme targeting hTERT mRNA showed stronger inhibition. Since the level of hTERT mRNA expression is less than that of hTR expression in cancer cells, hTERT might be a more useful therapeutic target[203]. An hTERT peptide vaccine GV1001 was tested in a phase I/II study of 48 patients with unresectable PC. The patients received intradermal injection in combination with GM-CSF. In the end, 24 of 38 evaluable patients demonstrated immune responses with the highest percentage (75%) in the intermediate dose group. Approximately 8.6 mo of mean survival for this group was significantly longer and one-year survival rate was 25%[152]. These promising results have led to commencement of another phase III trial of GV1001.

COX-2

COX-2 is a key enzyme of the metabolic process of arachidonic acid and an early response protein that is induced rapidly by growth factors, tumor promoters, oncogenes, and carcinogens. It plays an important role in tumorigenesis and angiogenesis. It has been shown that COX-2 mRNA and protein expression was highly upregulated in up to 90% of PC cases but was undetectable in nontumorous pancreatic tissue[204,205], and this indicated that COX-2 may be a potential target for treatment of PC. One study showed that COX-2 siRNA transfection could inhibit cell proliferation, induce cell apoptosis and regulate cell cycle of a PC cell line *in vitro* and decrease its tumorigenicity when inoculated subcutaneously into nude mice[206]. Nevertheless, inhibition of COX-2 is more applied in anti-inflammation treatment as nonsteroidal anti-inflammatory drugs (selective inhibitors of COX-2) than gene therapy.

Clinical research of gene therapy

Up to July 2013, 1970 gene therapy clinical trials have been completed, are ongoing or have been approved worldwide. Of these, 1264 (64.2%) have been made for treatment of cancers, including lung, gynecological, skin, urological, neurological and gastrointestinal tumors, as well as hematological malignancies and pediatric tumors. In this part, we summarized the finished and ongoing clinical trials of PC gene therapy worldwide (Tables 5 and 6) and the administration routes they employed (Figure 3). Meanwhile, since a variety of cancers have common characteristics, such as mutation of tumor suppressor genes or oncogenes and overexpression of tumor antigens, one therapeutic transgene can be effective for different tumors. In fact, many clinical trials are indicated for several cancers simultaneously, like all solid tumors

Table 5 Closed clinical trials of gene therapy in pancreatic cancer

Trial ID and investigator	Phase	Clinical indication	Patients (n)	Transgenes	Vectors and target cells	Administration route	Combined therapy	Results
DE-0009 Löhr et al[67]	I / II	Inoperable PC	14	Cytochrome p450	Naked/plasmid DNA transfected allogeneic human 293 embryonic kidney cells	Inject into the tumor vasculature via supraselective angiography	In combination with low-dose ifosfamide	4 patients showed tumor regression, the other ten individuals remained stable. Median survival was doubled compared with historic controls. 1-yr survival rate was three times better
DE-0024 Pecher et al[207]	I / II	Advanced breast cancer, PC and gallbladder carcinoma	10 (2 PC)	MUC-1	Naked/plasmid DNA transfected autologous dendritic cells	Subcutaneous injection	None	9 patients showed signs of progression. Only one remained stable for 3 mo until she was transferred to another therapy. 3 of 10 patients developed vaccine-specific delayed-type hypersensitivity reaction (DTH). 4 of 10 patients showed increased mucinspecific INF-gamma-secreting CD8+ T cells
DE-0063 Kubuschok et al[208]	I	PC	3 healthy donors and 1 PC patient	Mutated ras oncoprotein	EB virus transformed autologous lymphoblastoid cells	Subcutaneous injection	None	All the subjects showed strong vaccine-induced muRas-specific cytotoxic T lymphocytes
DE-0083 Niethammer et al[209]	I	Advanced PC	45	VEGFR-2	Naked/plasmid DNA (oral DNA vaccine)	Oral administration	In combination with gemcitabine	Not reported
ES-0004 Mazzolini et al[210]	I	Liver cancer, PC, colorectal cancer	17 (3 PC)	Interleukin-12 (IL-12)	Adenovirus transfected autologous dendritic cells	Intratumoral injection	None	Treatment was well tolerated. 11 of 17 were assessable for response. A partial response was observed in 1 case with PC. Stable disease was observed in 2 patients and progression in 8 patients
FR-0018 Gilly et al[211]	I / II	Unresectable digestive cancer	6 (3 PC)	Interleukin-2 (IL-2)	Adenovirus	Intratumoral injection	None	Good safety. But final results of tumor responses were not reported
US-0853 Le et al[212]	I	Ovarian cancer, PC, lung cancer, mesothelioma	28	Mesothelin	Listeria monocytogenes	Intravenous injection	1: Live attenuated Listeria vaccine (n = 9); 2: live attenuated mesothein expressing Listeria vaccine (n = 17)	In arm 2, Listeriolysin O and mesothelin-specific T-cell responses were seen and 37% of subjects lived ≥ 15 mo
US-0700 Galanis et al[213]	I	Gemcitabine-refractory, metastatic PC	12	Cyclin G1	Retrovirus	Intravenous injection	None	Good safety. But there was no evidence of anti-tumor activity
Kaufman et al[214]	I	Advanced PC	10	CEA, MUC-1, and TRICOM (including B7.1, ICAM-1, LFA-3)	Poxvirus	Subcutaneous injection	In combination with GM-CSF	Antigen-specific T-cell responses in 5 of 8 evaluated patients. 15.1 mo of median survival in responders vs 3.9 mo in non-responders. Overall median survival is 6.3 mo
Jaffee et al[141]	I	Resected PC	14	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	3 of 14 patients developed DTH, and 3 patients had increased disease-free survival time (at least 25 mo after diagnosis)
US-0475 Lutz et al[215]	II	Resected PC	60	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	In combination with standard chemoradiation	The median disease-free survival is 17.3 mo with median survival of 24.8 mo. Induction of CD8+ mesothelin-specific T cells correlated with disease-free survival
Laheru et al[142]	A pilot study	Advanced PC	50	GM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	A: vaccine alone (n = 20) B: vaccine plus cyclophosphamide (n = 30)	Cohort B showed enhanced mesothelin-specific T-cell responses. Median survival values in cohort A and cohort B were 2.3 and 4.3 mo
Le et al[143]	1 b	Advanced PC	30	CM-CSF	Naked/plasmid DNA transfected allogeneic tumor cell vaccine	Intradermal injection	1: Ipilimumab alone (n = 15); 2: Ipilimumab plus vaccine (n = 15)	Median overall survival was 3.6 mo for arm 1 and 5.7 mo for arm 2. Mesothelin-specific T cells responses were associated with increased disease-free survival in arm 2

Table 6 Ongoing clinical trials of gene therapy in pancreatic cancer

Phase	Transgenes
I	Oncolytic adenovirus and oncolytic herpesvirus[1]
	Somatostatin receptor 2 (sst2), Deoxycitidine kinase :: uridylmonophosphate kinase (dck::umk)
	Somatostatin receptor 2 (sst2)
	Mesothelin-scFv with signaling domains comprised of TCR , CD28, and 4-1BB (CD137) cDNA
	GM-CSF
	PANVAC (CEA, MUC-1, and TRICOM)
	Cytosine deaminase, Herpes simplex virus thymidine kinase (HSV-TK)
	Cyclin G1
	p53
	BikDD (DOTAP-cholesterol mediated gene transfection)
	Mutated Ras
I / II	Cytochrome p450
	AEG 35156: antisense oligonucleotide to X-linked inhibitor of apoptosis protein (XIAP)
	Herpes simplex virus thymidine kinase (HSV-TK)
	Diphtheria Toxin A Chain (DTA) gene with H19 promoter
	Alpha-(1,3) galactosyltransferase
II	CEA, MUC-1, and PANVAC
	Mutated Ras
	Alpha-(1,3) galactosyltransferase
III	Alpha-(1,3) galactosyltransferase
	PANVAC (CEA, MUC-1, and TRICOM)[2]

[1]Clinical trials of oncolytic viruses were completed or are ongoing in Spain and Japan, and their details were not clear; [2]This clinical trial has closed, but details and results have not been published yet.
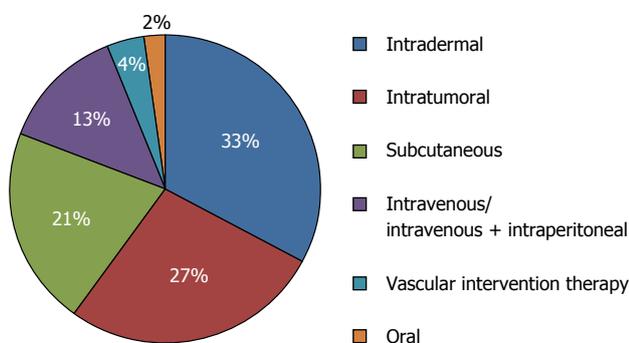
Figure 3 Clinical trials regarding administration routes of gene therapy in pancreatic cancer.

- Intradermal 33%
- Intratumoral 27%
- Subcutaneous 21%
- Intravenous/intravenous + intraperitoneal 13%
- Vascular intervention therapy 4%
- Oral 2%

or advanced tumors. Therefore, here we also collected such kind of trials and obtained a list of them (Table 7). It is worth noting that our analysis is mainly based on the records in The Journal of Gene Medicine Gene Therapy Clinical Trials Worldwide website (http://www.wiley.co.uk/genmed/clinical), so trials in some countries (*e.g.*, Japan) are not available.

Conclusions and prospects

Over the past decades, plenty of experimental works and clinical trials have demonstrated the safety and efficacy of cancer gene therapy. Strategies encompass tumor suppressor, suicide, anti-angiogenesis and immune related genes, as well as activated oncogenes and multidrug resistance genes. Proliferation and apoptosis related signal

Table 7 Clinical trials of unspecific tumor gene therapy that may involve pancreatic cancer

Phase	Clinical indication	Transgenes
II	Adenocarcinoma	CEA, TCRzeta and CD28
I	Cachexia	GHRH
I	CEA- or HER-2-expressing malignancies	HER-2, CEA
I	CEA-expressing malignancies	CEA
I / II	CEA-expressing malignancies	CAP-1 peptide from CEA
I / II	CEA-expressing malignancies	T cell receptor alpha and beta chains cDNA
I	CEA-expressing malignancies	CEA, B7.1, ICAM-1, LFA-3, GM-CSF
I	CEA-expressing malignancies	Anti-CEA-SFv-Zeta T cell receptor
I	CEA-expressing malignancies	B7.1 (CD80)
II	Advanced cancer with overexpression of p53	Anti-p53 T cell receptor
I	MUC-1- expressing tumors	MUC-1, IL-2
I	Advanced cancer	Cytochrome P450
I	Advanced cancer	Endostatin
I	Advanced cancer	Heat shock protein 70
I	Advanced cancer	T cell receptor alpha and beta chain
I	Advanced cancer	Bifunctional shRNA specific for stathmin 1 oncoprotein
I / II	Advanced cancer	GM-CSF
I	Advanced cancer	IL-12
I / II	Advanced cancer	CYP1B1
I	Advanced cancer	GM-CSF, CD154 (CD40-ligand)
I / II	Advanced cancer	IL-2
I	Advanced cancer	p53
I	Advanced cancer	AMEP
I	Advanced cancer	B7.1 (CD80) ,ICAM-1, LFA-3
I / II	Advanced cancer	Cytosine deaminase
I / II	Advanced cancer	CEA
I	Solid tumors	Tumor antigen
I	Solid tumors	Interferon-gamma
I	Solid tumors	TNF
I	Solid tumors	Brachyury oncoprotein
I	Solid tumors	Human telomerase reverse transcriptase
I / II	Solid tumors	Oncolytic virus (no transgene)
I	Solid tumors	Retinoblastoma 94
I	Solid tumors	p53
I	Solid tumors	O6-methylguanine DNA methyltransferase (MGMT)
I	Solid tumors	GM-CSF
I	Solid tumors	GM-CSF, bi-shRNA-furin
I	Solid tumors	GM-CSF, TGF-beta 2 antisense
I	Solid tumors	GM-CSF, humanized *Escherichia coli* beta-galactosidase

CEA: Carcinoembryonic antigen; IL-2: Interleukin-2; AMEP: Antiangiogenic metargidin peptide; CYP1B1: Cytochrome P450 isoenzyme 1B1; TNF: Tumor necrosis factor; hTERT: Human telomerase reverse transcriptase; GHRH: Human growth hormone releasing hormone.

pathways are also possible targets. However, among these therapeutic targets, only a small part were tested in clinical settings and proved to be effective, and even for them, outcomes were far from curative and treatments have to be combined with standard chemo- or radio-therapy for maximum benefits.

In this field, tumor suppressor gene *p53* and mutant

oncogene *K-ras* are two members that have been most deeply studied in all kinds of cancers and have yielded encouraging results *in vitro* and in animal models. Unfortunately, these findings have not been translated to improve outcomes in clinical trials. In suicide strategy, HSV-TK, CD and cytochrome p450 have been proved to be efficacious. A closed clinical trial of cytochrome p450 showed exciting results when combined with ifosfamide, and more trials are ongoing. As to anti-angiogenesis approach, VEGFR is of course the best choice, clinical trials of peptide vaccine derived from VEGFR-2 and DNA vaccine targeting VEGFR-2 did show benefits, but as a monotherapy it is not a "magic bullet" for all tumor patients since anti-angiogenesis is far more effective in preventing tumor growth than causing regression of established tumors, thus this method may be more suitable for patients with minimal residual disease. Nowadays immunotherapy has become the mainstream direction of studies in searching new cancer treatment regimens, and the cooperation of gene therapy and immunotherapy has created even more inspiring achievements, such as adoptive transfer of genetically modified T-cells or DCs, DNA vaccines (CEA, MUC-1, B7.1, ICAM-1, LFA-3), and direct transfer of cytokine genes (IL-2, IL-12, GM-CSF, INF-gamma). Of course, combining different targets or combination of gene therapy and traditional chemoradiotherapy may lead to improved efficacy of gene therapy in PC.

Considering that low efficiency of gene transfer is the main obstacle in application and popularization of gene therapy, the selection of high-efficient and tumor-targeted vectors should be emphasized. In general, viral vectors offer higher transduction efficiency and long-term gene expression and this is why more than two-thirds of clinical trials reported employ viral vectors. Recently, oncolytic viruses that infect and replicate selectively in cancer cells have become the hotspot in this field and show a bright future. Oncolytic viruses produced from initially infected cancer cells can spread to surrounding cancer tissue and distant tissue, thus intratumoural injections could be efficient, even for the treatment of disseminated tumors. Systemic administration of oncolytic viruses has also shown good safety and this is of great interest particularly in PC, since it is difficult to inject viruses into primary lesions directly in PC patients.

Gene therapy of cancer is not yet applied in routine clinical practice, but good safety records and validated clinical benefits make it a good candidate for all clinical settings including neo-adjuvant, adjuvant and palliative treatment. Despite these promising therapeutic strategies, greatest advance in the treatment of PC may still come from improved early detection and diagnosis.

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2014 ADVANCES IN PANCREATIC CANCER

Pathology of pancreatic ductal adenocarcinoma: Facts, challenges and future developments

Irene Esposito, Björn Konukiewitz, Anna Melissa Schlitter, Günter Klöppel

Irene Esposito, Björn Konukiewitz, Anna Melissa Schlitter, Günter Klöppel, Institute of Pathology, Technische Universität München, 81675 Munich, Germany
Author contributions: Esposito I, Konukiewitz B and Schlitter AM contributed to the study idea, study design, literature search, manuscript writing and final revision of the article; Klöppel G contributed to the study idea, study design and final revision of the article.
Correspondence to: Irene Esposito, MD, Institute of Pathology, Technische Universität München, Ismaningerstr 22, 81675 Munich, Germany. esposito@lrz.tu-muenchen.de
Telephone: +49-89-41404166 Fax: +49-89-41404865

Abstract

Despite major improvements concerning its diagnosis and treatment, pancreatic ductal adenocarcinoma (PDAC) remains an aggressive disease with an extremely poor prognosis. Pathology, as interface discipline between basic and clinical medicine, has substantially contributed to the recent developments and has laid the basis for further progress. The definition and classification of precursor lesions of PDAC and their molecular characterization is a fundamental step for the potential identification of biomarkers and the development of imaging methods for early detection. In addition, by integrating findings in humans with the knowledge acquired through the investigation of transgenic mouse models for PDAC, a new model for pancreatic carcinogenesis has been proposed and partially validated in individuals with genetic predisposition for PDAC. The introduction and validation of a standardized system for pathology reporting based on the axial slicing technique has shown that most pancreatic can-

cer resections are R1 resections and that this is due to inherent anatomical and biological properties of PDAC. This standardized assessment of prognostic relevant parameters represents the basis for the successful conduction of multicentric studies and for the interpretation of their results. Finally, recent studies have shown that distinct molecular subtypes of PDAC exist and are associated with different prognosis and therapy response. The prospective validation of these results and the integration of molecular analyses in a comprehensive pathology report in the context of individualised cancer therapy represent a major challenge for the future.

Core tip: Despite recent progresses, pancreatic ductal adenocarcinoma (PDAC) remains a disease with poor prognosis. Pathology has given fundamental contributions to these developments. In particular, precursor lesions have been identified and a model for PDAC development has been proposed and validated by molecular studies, which represent the basis for the identification of biomarkers for early diagnosis. A standardized protocol for the post-operative assessment of prognostic relevant parameters, such as the resection margin status, has been developed and has shown a high degree of interlaboratory reproducibility. Finally, the genome-wide analysis of PDAC has led to the identification of distinct molecular subtypes with different therapy response and clinical courses.

INTRODUCTION

In the last two decades major improvements in the understanding of pancreatic ductal adenocarcinoma (PDAC) have been achieved by the scientific community, with extraordinary contributions from all disciplines of life sciences, from genetics and molecular biology to molecular imaging and oncology[1,2]. Milestones of this continuing evolving process are for instance the definition and classification of the precursor lesions of PDAC, such as the microscopic pancreatic intraepithelial neoplasia (PanIN)[3] and the larger intraductal papillary mucinous neoplasm (IPMN), which have paved the way for further studies concerning the natural history[4] and the molecular characterization of these lesions, leading to the exploitation of new and more sensitive imaging methods for the purpose of early diagnosis[5]. The isolation and characterization of pancreatic stellate cells[6,7] and hereby the identification of the cancer-associated desmoplastic reaction as an active player in affecting deleterious properties of PDAC, such as its migratory and invasive ability and its capability to adapt to a hypoxic microenvironment[8-10] have also been subjects of intense research activities in the last years. In 2003 the first transgenic mouse model that faithfully recapitulates the development of PDAC from low-grade precursors (so-called PanIN1) to metastatic cancer has been generated and rendered available to the scientific community[11]. Numerous further mouse models have been generated and characterized ever since[12]. These models represent useful instruments to improve our knowledge of the molecular mechanisms and the cellular interactions regulating PDAC initiation and progression, as well as providing an indispensable platform for the development of molecular tracers[13] and for drug testing[14,15]. Following the complete sequencing of the human genome[16] and the advancement of high-throughput molecular methods, the genetic complexity of PDAC has been addressed, culminating in the sequencing of its genome and the identification of the most relevant genetic alterations and molecular pathways of this disease[17]. Pathology (with its branches of anatomical, clinical and molecular pathology) represents the interface between basic research and clinical medicine and facilitates translational research. As such, pathology has been playing a major role in all the above described relevant steps and achievements. In this review, the major contributions of pathology to the improvement of our knowledge and understanding of PDAC in recent years will be addressed, with special focus on clinically relevant, innovative aspects and future challenges.

PRECURSOR LESIONS: EARLY DETECTION OF PANCREATIC CANCER

Morphology and genetics

The classical and well-characterized precursor lesions of PDAC show a ductal phenotype, suggesting a ductal cell of origin of this tumor. The most frequent precursors are PanIN, followed by IPMN and mucinous cystic neoplasms (MCN). PanIN are microscopical (< 5 mm) mucinous-papillary lesions, which lead to invasive carcinoma through an adenoma-carcinoma sequence, in analogy to the Vogelstein's model of colon carcinogenesis[18]. Accordingly, using modern methods of pyrosequencing and high-resolution melt-curve analysis, it has been shown that virtually all PanIN, including more than 90% of low-grade PanIN, harbor mutations in the *KRAS* gene locus, followed by *CDKN2A/p16*, *SMAD4* and *TP53* mutations in intermediate and later stages of pancreatic carcinogenesis[19-21]. Similarly, both IPMN and MCN give rise to invasive PDAC by stepwise gene alterations. IPMN are the most frequent cystic neoplasms in surgical series and show a different malignant potential depending on their site of origin (main pancreatic duct *vs* side-branch duct) and their histological subtype[22,23]. The main histopathological, immunophenotypical and clinical characteristics of IPMN are summarized in Table 1. *KRAS* and *GNAS1* mutations[24], represent early genetic alterations whereas *TP53* mutations represent late changes in the adenoma-carcinoma sequence of IPMN leading to invasive cancer[20,22,25]. Different histological IPMN subtypes have been associated with different frequency of mutations, with *KRAS* mutations being particularly associated with the gastric subtype and *GNAS1* mutations with the intestinal subtype[24,26]. These different molecular changes probably reflect different pathways of cancer progression. For example it has been reported that *KRAS*-mutated PDAC often arises in pancreata with low-grade gastric type IPMN, whereas *GNAS1*-associated intestinal type IPMN are often high-grade lesions which can develop into invasive carcinoma of colloid type[27]. Despite these differences, altogether these data indicate the ductal phenotype and the presence of *KRAS* mutations as common characteristics of PDAC precursors.

The generation of transgenic mouse models that closely reproduce the human PanIN/IPMN-PDAC sequence have on one side confirmed the relevance of *KRAS* as driver gene in PDAC (for reviews about mouse models for PDAC and the role of *KRAS*[12,28]). On the other side, the ductal origin of PDAC has been challenged, since targeting the ductal compartment by genetic manipulation has failed to generate PanIN and PDAC so far, with the exception of a few PanIN1-like lesions[29-31]. Early progenitor cells, as well as exocrine progenitors and

Table 1 Histopathological, immunophenotypical and clinical characteristics of intraductal papillary mucinous neoplasm (adapted from [22])

Types and subtypes	Main localization	Sex	Frequency	Microscopic findings	Immunophenotype					Association with PDAC
					MUC1	MUC2	MUC5AC	MUC6	CDX2	
Main-duct type IPMN										
Intestinal type	Pancreatic head	M(>)F	36%	Papillae lined by columnar cells	-	+	+	-	+	34%
Pancreatobiliary type	Pancreatic head	M(>)F	7%	Papillae lined by cuboidal cells	+	-	+	+	-	58%
Oncocytic type	Pancreatic head	M(>)F	9%	Complexe papillae lined by multiple layer of cells with eosinophilic cytoplasma	+	-	+	+	-	25%
Branch-duct type IPMN										
Gastric type	Uncinate process	M(>)F	49%	Gastric foveolar epithelium	-	-	+	-	-	6%

IPMN: Intraductal papillary mucinous neoplasm; PDAC: Pancreatic ductal adenocarcinoma.
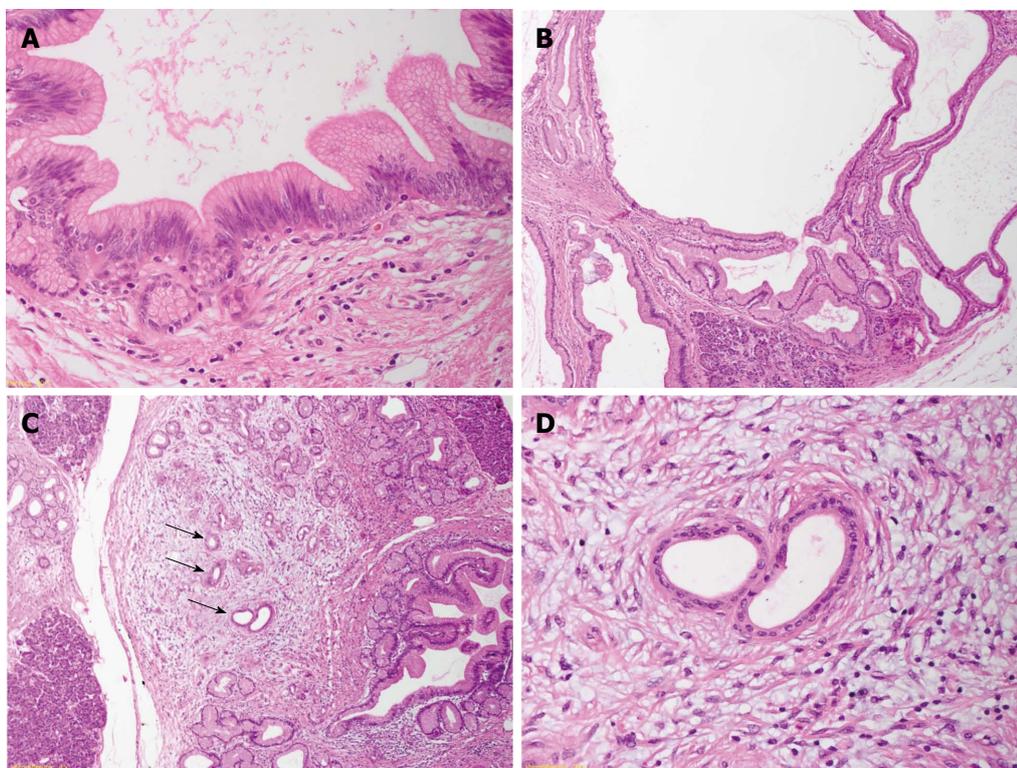
Figure 1 Precursor lesions of pancreatic ductal adenocarcinoma in familial pancreatic cancer-patients. A: Low-grade pancreatic intraepithelial neoplasia; B: Gastric-type intraductal papillary mucinous neoplasm showing a ductal phenotype; C: Acinar-ductal metaplasia in areas of lobulocentric atrophy. Arrows indicate an atypical flat lesion; D: Atypical flat lesion with cellular atypia and typical stromal reaction.

even adult acinar and insulin-producing cells can instead be targeted to generate PanIN lesions thus closely reproducing the development of human PDAC[32]. These studies have raised the hypothesis of an alternative model of pancreatic carcinogenesis, which starts from the centro-acinar-acinar compartment and develops into PanIN and PDAC through a metaplasia-dysplasia sequence[33] (Figure 1). According to recent data, it seems now plausible that PDAC can directly originate from the centroacinar-acinar compartment without the intermediate step of PanIN, according to the so-called AFL- (or acinar-ductal) carcinogenesis model[34,35] (Figure 2). The morphological correlate of this model is the atypical flat lesion (AFL). AFL represents the most probable precursor lesion of PDAC in the Kras$^{G12D/+}$; Ptf1a-Cre$^{ex1/+}$ mouse model. AFL are localized in the centroacinar-acinar compartment and display a ductal phenotype, thus potentially originating through a process of acinar-ductal metaplasia. AFL consists of a CK19-positive flat to cuboidal epithelium with enlarged nuclei, an increased nuclear to cytoplasmic ratio, prominent nucleoli and evident mitotic figures. The Ki-67 index is elevated; *CDKN2A/p16* is altered through hyper-
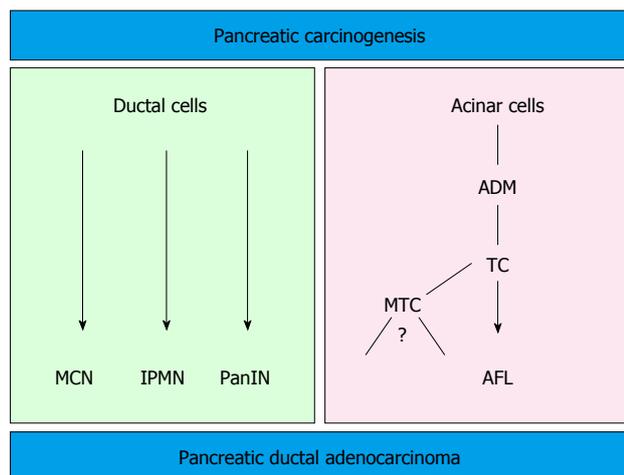
Figure 2 Dual model of pancreatic carcinogenesis. The well-known precursor lesions (PanIN, IPMN and MCN) show a ductal phenotype. However, it seems now plausible that pancreatic ductal adenocarcinoma can directly originate from the centroacinar-acinar compartment through atypical flat lesions without the intermediate step of pancreatic intraepithelial neoplasia (PanIN) (acinar-ductal carcinogenesis). MCN: Mucinous cystic neoplasm; IPMN: Intraductal papillary mucinous neoplasm; ADM: Acinar ductal metaplasia; TC: Tubular complexes; MTC: Mucinous tubular complexes; AFL: Atypical flat lesion.

methylation of the corresponding promoter or by intragenic deletion. The surrounding stroma shows a selective overexpression of α-smooth muscle actin, indicating a local activation like that seen in invasive carcinomas[34].

Relevance for the clinic

The relevance of the above described carcinogenesis models has been confirmed by studying the pancreata of individuals with an elevated risk of developing PDAC during their life. An estimated 10% of all PDAC show a familial background. Several genetic syndromes (Table 2) are known to be associated with an elevated life-time risk for the development of PDAC[25,36-38]. However, specific gene mutations that account for the majority of cases, grouped under the term "familial pancreatic cancer" (FPC), have not been identified. FPC has been described as an autosomal dominant inheritance with high penetrance[37] and the pathology of resection specimens of FPC individuals has been recently described. Ductal precursor lesions such as PanIN and IPMN, especially gastric type IPMN, are a common finding. In comparison with sporadic disease, the number of precursor lesions is higher, they are usually multifocal throughout the organ and they display a higher grade of dysplasia[36,39,40]. Another important finding associated with multifocal PanIN and IPMN is the lobulocentric atrophy[25], a multifocal change consisting of acinar atrophy, fibrosis and acinar-ductal metaplasia. Lesions similar to murine AFL have been recently described in areas of lobulocentric atrophy of FPC individuals undergoing prophylactic pancreatectomy and represent the first evidence of the existence of an AFL-carcinogenesis pathway in humans in addition to the established PanIN/IPMN pathway (Figure 1)[34]. Since lobulocentric atrophy can be identified by endo-

Table 2 Genetic syndromes that are associated with an elevated lifetime risk for the development of pancreatic ductal adenocarcinoma (adapted from [25])

Syndrome	Gene
Familial breast cancer	*BRCA2*
FAMMM syndrome	*CDKN2A/p16*
Lynch syndrome	*MSH2, MLH1, others*
Heriditary pancreatitis	*PRSS1*
Peutz-Jeghers syndrome	*STK11*
Ataxia teleangiectatica	*ATM*

scopic ultrasound and magnetic resonance tomography, its strong association with PDAC precursors can be used to identify high-risk individuals and to monitor FPC patients in future screening programs.

Future challenges

The relevance of the described changes for sporadic PDAC has yet to be fully investigated, although it is known that not only PanIN, but also acinar-ductal metaplasia is a common finding in the pancreas of adult patients, independently from the underlying disease[41].

Future studies should aim at further characterizing AFL from the molecular point of view, in order to define specific genetic signatures that may on one side strengthen the model of AFL-carcinogenesis and on the other side help in identifying biomarkers for early detection.

ANATOMICAL AND SURGICAL PATHOLOGY: STANDARDIZED PATHOLOGICAL REPORTING FOR A MORE ACCURATE DEFINITION OF PROGNOSTIC RELEVANT PARAMETERS

Axial slicing technique and the 1-mm rule

The pathology examination procedure plays an important role in the diagnostic and classification of PDAC. The axial slicing technique includes a standardized inking of the fresh or fixed resection specimen according to a predefined color code followed by axial slicing perpendicular to the longitudinal axis of the descending duodenum, as previously described[42,43]. After a correct orientation of the specimen, which should follow in close collaboration with the surgical team (*i.e.*, directly in the operating theater or, if not possible, by marking relevant anatomic landmarks with loose sutures), this method is rapid and easy to perform. Moreover, it offers technical advantages compared to other slicing methods, such as the opening of the specimen in a longitudinal manner by exposing the main pancreatic and the bile duct. In fact, the axial slice technique does not depend on the configuration of the pancreatic and the bile duct and it is not impaired by duct obstructions, which are a relative common finding in PDAC resections[44]. All relevant anatomic structures and their relationship to the tumor are easily displayed, the
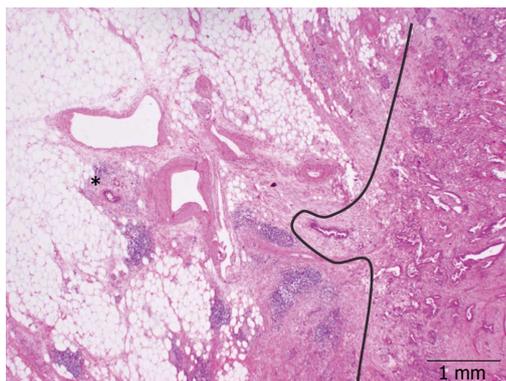
Figure 3 Dispersed growth of pancreatic ductal adenocarcinoma. Tumor deposits (star) of pancreatic ductal adenocarcinoma are found many mm away from the main tumor bulk. The curved line highlights the tumor front.

resection margins remain intact before slicing and their distance from the tumor can be easily measured after perpendicular sampling[42].

A striking characteristic of PDAC is its very dispersed and infiltrative type of growth, particularly evident at the tumor periphery, where tumor deposits may be found many mm away from the main tumor bulk (Figure 3). This simple morphological observation has been substantiated by a recent study where the minimum spanning tree analysis was used to calculate tumor cell dispersion in the tumor center and periphery[45]. The following logic consequence of this observation has been the introduction of the so-called 1-mm rule for the assessment of margin clearance in PDAC: in analogy to what has already been validated for the assessment of mesorectal clearance in rectal cancer, a pancreatic resection margin is defined as free (R0) if the tumor cells lies ≥ 1 mm from the margin itself[42,44].

Relevance for the clinic

It has been shown that the rate of microscopic margin involvement (R-status) is related to the different grossing techniques. In particular, the use of the axial slicing technique and the application of the 1-mm rule lead to a significant higher rate of R1 resections compared to other techniques, with higher reproducibility and smaller deviation of this and other morphology-based parameters[42-44,46-50]. These results bear three important consequences. First of all, they highlight the fact that a high frequency of R1 resections for PDAC is not linked to the surgical techniques but mostly depend on high-quality pathology examination[42]. Second, a standardized pathology report based on a highly reproducible slicing technique allows a fair comparison between results from different institutions, which represents the basis for the execution of multicentric studies[51]. Third, the R-status assessed according to the axial slicing technique becomes a prognostic relevant factor at multivariate analysis that can be used to tailor post-operative treatments[50] according to the concepts of individualized medicine.

Future challenges

The 1-mm rule, although based on morphological evidence of a dispersed type of growth in PDAC, was introduced arbitrarily, mostly in analogy to mesorectal margin assessment in rectal cancer. Some studies have suggested that wider clearances are needed[50,52]. This aspect should be carefully evaluated in prospective studies, in order to define optimal margin clearance for PDAC.

MOLECULAR SUBTYPES OF PDAC: TOWARDS PERSONALIZED CANCER MANAGEMENT

Molecular genetics of PDAC

Recent advances in pancreatic cancer biology have led to the discovery of recurrent gene mutations in *KRAS*, *SMAD4*, *TP53* and *CDKN2A/p16*, the identification of core signalling pathways and the definition of molecular subtypes with distinct prognosis and therapy response. In 2008, the first global genomic analysis of 24 advanced PDAC identified 12 core signalling pathways that are genetically altered in the great majority of PDAC. These include apoptosis, DNA damage control, Hedgehog and Wnt/Notch signalling pathways, among others[17]. Interestingly, the affected genes within these pathways highly differed between individual tumors. An average of 63 genetic alterations was found in the cancer cells, most commonly point mutations[17,53]. The new concept that mutations in PDAC are frequent and not restricted to single genes has important implications for treatment strategies: Firstly, the need to target key points and downstream targets of core signalling pathways rather that a single targetable oncogene/tumor suppressor gene. Secondly, the identification of less characterized signalling pathways in PDAC as additional drug targets (*e.g.*, Hedgehog and integrin pathways)[17].

Relevance for the clinic

In 2011, Collisson *et al*[54] defined three distinct PDAC subtypes based on combined analysis of transcriptional profiles of primary tumor samples and human and mouse PDAC cell lines: classical, quasi-mesenchymal and exocrine-like subtypes. The authors found that stratification by subtype provided prognostic information reflected by the significantly better survival of individuals with classical subtype compared to individuals with quasi-mesenchymal subtype. Further analyses of human PDAC cell lines of known subtype suggested that drug responses differ by subtype: targeted therapies (erlotinib) were more effective in classical subtype, whereas cancer cell lines of the quasi-mesenchymal subtype were more sensitive to conventional chemotherapy (gemcitabine). In addition, targeting inactivated *KRAS* in classical and quasi-mesenchymal cell lines using RNA interference showed significantly more antiproliferative effect in the classical cell type compared to quasi-mesenchymal cell
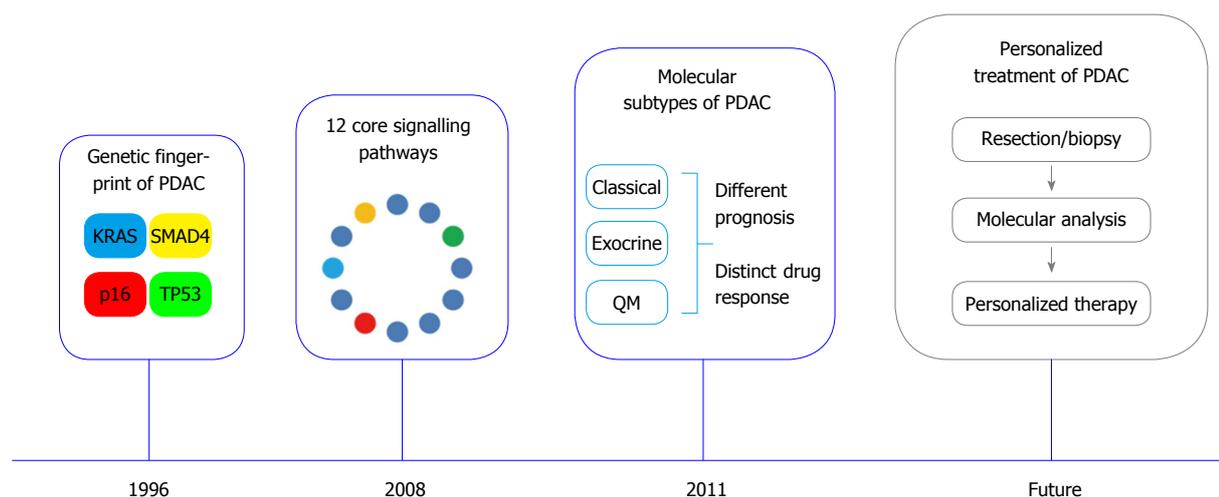
Figure 4 Milestones in pancreatic cancer biology. Mutations of *KRAS*, *CDKN2A/p16*, *SMAD4* and *TP53* represent the molecular fingerprint of pancreatic ductal adenocarcinoma (PDAC)[53]. Global genomic analysis of PDAC defined a set of 12 core signalling pathways commonly altered in the great majority of investigated tumors[17]. Three distinct molecular PDAC subtypes ["classical", "quasi-mesenchymal" (QM) and "exocrine-like"] with prognostic relevance and distinct drug response were defined based on combined analysis of transcriptional profiles of primary tumor samples and human and mouse PDAC cell lines[54].

lines[54]. Likewise, the effect of concurrent inhibition of MEK, a downstream target of KRAS, and EGFR was demonstrated to be restricted to epithelial/classical subtype in a recent study[55]. The clinical relevance of the exocrine subtype, a subtype characterized by high expression of digestive enzymes, remains questionable. Although the exocrine subtype was identified in primary tumor samples, no representative tumor cell line could be identified among the investigated cell line collection, raising the possibility of an contamination artifact with normal pancreatic tissue[54].

The relationship between the four most common mutations of PDAC and the proposed molecular subtypes is largely unknown. However, it is increasingly clear that mutations in *KRAS*, *SMAD4*, *TP53* and *CDKN2A/p16* are "driver" mutations of PDAC, *i.e.*, mutations that confer a selective growth advantage to the tumor cell[56], and they are key players within a complex, and not yet fully understood, network of core pathways[57].

The clinical significance of these driver mutations and intact core pathways has been highlighted by several publications. Among the four driver mutations, *KRAS* is most commonly mutated in PDAC and exhibits mutations rates up to 95%[58]. Although rare, PDAC patients with non-mutated *KRAS* tend to have a significantly better median survival than patients with mutated *KRAS*[59]. Yachida *et al*[60] investigated the mutation status of *KRAS*, *TP53*, *CDKN2A/p16* and *SMAD4* in an autopsy study of 79 patients and showed that the number of altered genes correlates with a significantly better overall and disease free survival and more indolent disease in patients with only 1 or 2 driver mutations.

Future challenges

These data suggest that the mutation status of *KRAS*, *TP53*, *CDKN2A/p16* and *SMAD4*, also referred as the genetic fingerprint of PDAC, might be an integral part

of a comprehensive pathology report in the future (Figure 4). Taken together, definition of altered major molecular pathways in PDAC and identification of molecular subtypes have allowed stratification of patients into groups with different biological behaviors.

CONCLUSION

Despite enormous progresses during the last two decades, PDAC remains a disease with still high mortality rates. Pathology plays a fundamental role as interface discipline between basic research and clinic in improving our knowledge about the development of the disease and in providing tools to improve early diagnosis and personalized treatment. The morphological and molecular characterization of the precursor lesions may help in identifying biomarkers of early disease that could be first tested in high risk individuals and then validated in the general population. The introduction of a highly reproducible standardized pathological reporting method based on the axial slicing technique allows an exact definition of prognostic relevant parameters, such as the R-status, and represents the basis for the comparison of results obtained from multicentric clinical studies. Finally, the foreseeable identification of molecular subtypes of PDAC with different clinical behavior and response to therapy and the integration of these data into routine histopathological diagnosis will help to individualize the treatment of PDAC (Figure 4).

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2014 ADVANCES IN PANCREATIC CANCER

Update on surgical treatment of pancreatic neuroendocrine neoplasms

Jan G D'Haese, Chiara Tosolini, Güralp O Ceyhan, Bo Kong, Irene Esposito, Christoph W Michalski, Jörg Kleeff

Jan G D'Haese, Chiara Tosolini, Güralp O Ceyhan, Bo Kong, Christoph W Michalski, Jörg Kleeff, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, 81675 Munich, Germany
Irene Esposito, Institute of Pathology, Technische Universität München, 81675 Munich, Germany
Author contributions: All authors contributed to the manuscript.
Correspondence to: Jörg Kleeff, MD, Department of Surgery, Klinikum rechts der Isar, Technische Universität München, Ismaningerstrasse 22, 81675 Munich, Germany. kleeff@tum.de
Telephone: +49-89-41405098 Fax: +49-89-41405098

Abstract

Pancreatic neuroendocrine neoplasms (PNENs) are rare and account for only 2%-4% of all pancreatic neoplasms. All PNENs are potential (neurendocrine tumors PNETs) or overt (neuroendocrine carcinomas PNECs) malignant, but a subset of PNETs is low-risk. Even in case of low-risk PNETs surgical resection is frequently required to treat hormone-related symptoms and to obtain an appropriate pathological diagnosis. Low-risk PNETs in the body and the tail are ideal for minimally-invasive approaches which should be tailored to the individual patient. Generally, surgeons must aim for parenchyma sparing in these cases. In high-risk and malignant PNENs, indications for tumor resection are much wider than for pancreatic adenocarcinoma, in many cases due to the relatively benign tumor biology. Thus, patients with locally advanced and meta-static PNETs may benefit from extensive resection. In experienced hands, even multi-organ resections are accomplished with acceptable perioperative morbidity and mortality rates and are associated with excellent long term survival. However, poorly differentiated neoplasms with high proliferation rates are associated with a dismal prognosis and may frequently only be treated with chemotherapy. The evidence on surgical treatment of PNENs stems from reviews of mostly single-center series and some analyses of nation-wide tumor registries. No randomized trial has been performed to compare surgical and non-surgical therapies in potentially resectable PNEN. Though such a trial would principally be desirable, ethical considerations and the heterogeneity of PNENs preclude realization of such a study. In the current review, we summarize recent advances in the surgical treatment of PNENs.

Core tip: Surgical resection is the only curative treatment for pancreatic neuroendocrine neoplasms (PNENs). Surgical resection should be tailored and parenchyma-preserving whenever possible. Laparoscopic approaches are feasible and safe for pancreatic body and tail lesions. Regional lymph node dissection may prolong disease free survival. Cytoreductive surgery and palliative debulking (> 90%) of PNET liver metastases may extend survival. The most relevant prognostic factors are surgical intervention, tumor differentiation, patient age, and distant metastases.

INTRODUCTION

Pancreatic neuroendocrine neoplasms (PNENs) are rare with an incidence of about 1/100000 per year causing

only 1%-4% of all clinically apparent pancreatic neo-plasms[1-3]. Most PNENs are sporadic but about 10% are part of inherited disorders such as multiple endocrine neoplasia type 1, von Hippel-Lindau syndrome, neurofi-bromatosis and tuberous sclerosis.

PNENs seem to arise from the islet cells of the pancreas and may or may not secrete functionally active hormones and can therefore be classified as functional or non-functional tumors. Functional tumors are usually detected early due to the symptoms caused by hormone production. Recent studies suggest that most PNENs are non-functional and therefore diagnosed either incidentally or late due to unspecific symptoms caused by the local or distant tumor mass. However, the traditional distinction between functional and non-functional tumors has become clinically largely irrelevant since this distinction does not influence prognosis or treatment options.

A clinically much more relevant classification is the generally accepted grading of PNENs on the basis of the 2010 WHO classification for gastroenteropancreatic neuroendocrine tumors and the expression of the cell proliferation marker Ki-67. Accordingly, PNENs are graded as G1 [mitotic count < 2/10 high power fields (HPF) and a Ki-67 index < 3 %], G2 (mitotic count of 2-20/10 HPF and a Ki67 index of 3%-20%), and G3 (mitotic count > 20/10 HPF and/or a KI-67 index > 20%). Differentiation on the other hand refers to the extent to which the neoplastic cells resemble their non-neoplastic counterparts[4]. In general, well differentiated PNENs are either low or intermediate grade (G1 + G2) and are termed neuroendocrine tumors (PNETs), while poorly differentiated PNENs are considered high grade (G3) and are called neuroendocrine carcinomas (PNECs).

The differential diagnosis between PNENs and pancreatic ductal adenocarcinoma (PDAC) is especially important because major differences in tumor biology require different surgical treatment strategies. Since PNET patients have a much better prognosis than PDAC patients, surgery is more frequently the treatment of choice. This review will provide an update on current surgical treatment options for patients with PNENs.

SURGICAL TREATMENT OF PNETS

Surgical resection of PNETs remains the only curative approach and must therefore be regarded as the current standard of care even in many cases where advanced disease is found[5-7]. However, only about two thirds of the patients present with technically resectable disease. Tailored surgical approaches are therefore needed to deal with this very heterogenic disease.

Management of low risk disease

PNETs show a benign biological behavior in 10%-40%, most of them being insulinomas[8]. If benign PNETs are solitary and easily accessible, local resection/enucleation is generally preferred[9]. In this respect, a recent study demonstrated the importance of intraoperative bi-digital

palpation and ultrasonography (IOUS) in localizing these lesions[10]. Besides, IOUS is useful in clarifying the association of the tumor lesions, the pancreatic vasculature and particularly, the main pancreatic duct. Therefore, it provides important information in deciding between enucleation and resection[10]. When the tumor is located further than 2-3 mm from the pancreatic duct, an enucleation is generally preferred to pancreatic resection[5]. Furthermore, preoperative endoscopic tattooing of lesions in the pancreatic head or tail seems to be a feasible alternative for intraoperative localization of the tumor especially for laparoscopic surgical procedures[11]. When enucleation seems possible, the tumor is carefully dissected off the surrounding pancreatic tissue[12]. After resection, a drain may be placed at the resection site[12]. As an alternative in cases where enucleation seems impossible, middle segmental pancreatic resection may be performed as a parenchyma-preserving technique[13]. Such organ preserving strategies are nowadays safe in experienced hands with low morbidity and mortality[13,14]. Although parenchyma-preserving techniques have slightly increased morbidities (76%) and pancreatic fistula (69%) compared to standard resections (58% and 42%), the patients do clearly benefit in terms of pancreatic endo- and exocrine function[14]. Organ preserving pancreatic surgery leads to only 3%-5% impairment of endo- and exocrine function, whereas in standard resections this rate can increase up to 21%-32%[14]. Furthermore it seems evident, that while performing organ preserving and locally limited surgery the patients are not put at risk concerning postoperative survival compared to standard resections[14].

Laparoscopic resection seems to be ideal for insulinomas that are usually benign, small, and located in the body or tail of the pancreas; this procedure has been shown to carry a low risk of morbidity and mortality[15,16]. However, because of the difficult preoperative assessment of the nature and extension of the tumor, a conversion to open surgery is frequent. Recently, robot-assisted minimally-invasive pancreatic resections have been suggested to be superior to the laparoscopic approach since conversions to open surgery can be significantly reduced (conversion rate of 0% *vs* 16%) without increased morbidity[17]. The robot-inherent disadvantages of a lack of haptics, a steep learning curve and high costs however prevent many centers from implementing this technique. Nevertheless, in the presence of tumors with a high probability of malignancy, or in the absence of a cleavage plane to duct and blood vessels, open surgery may be considered in the first place[12].

There is an ongoing debate on the role of lymph node dissection in PNEN surgery. When considering organ preserving surgery for low risk PNENs other than insulinomas, recent data showing a positive lymph node status in up to 23% of low risk PNENs with significantly shorter disease free survival (mean 4.5 years *vs* 14.6 years; *P* < 0.0001) should be considered[18]. The frequency of lymph node metastases was reported to be higher for tumors > 15 mm, tumors in the head as compared to tumors in the

body and the tail, tumors with higher proliferation rates (G3), and with lymph vessel invasion (L1)[18,19]. Partelli and colleagues developed two predictive models to assess the risk of positive lymph nodes in non-functional PNENs, one with histopathological grading and one without[20]. In addition to the previously mentioned factors, radiological nodal status was associated with lymph node metastases in their study[20]. However, considering current evidence, it seems that preoperative variables are not able to predict the probability of nodal involvement sufficiently enough to omit regional lymphadenectomy. Therefore, regional lymphadenectomy is suggested for patients undergoing pancreatic resections for PNENs.

Management of high risk/malignant disease

In case of malignancy, recent studies proved that extensive surgery is superior to conservative therapies in extending patients' survival and in controlling local and metastatic disease.

Early and locally advanced disease

In case of localized tumors, the aim of surgery is to achieve curative resection and to prevent or delay local or metastatic recurrence. Here, oncological resections (partial pancreaticoduodenectomy or distal pancreatic resection) are required. A recent study showed a survival benefit of 79 mo for resected patients compared to those patients who were recommended for but did not undergo resection (114 mo *vs* 35 mo; $P < 0.0001$)[21]. However, one should note that in this study patients that were recommended for but did not undergo resection showed considerably more often distant metastases when compared to the group of resected patients (58.3% *vs* 28.4%). Nevertheless, the survival advantage of resection appeared to hold true also for the subgroup of patients with distant metastases (60 mo *vs* 31 mo, $P = 0.01$). Even though these data are retrospective, they suggest an impressive benefit of surgical resection in extending survival. Furthermore, resection has been shown to reduce the risk for the development of metachronous liver metastases. Patients with gastrinoma that underwent surgical resection developed significantly less metachronous liver metastasis (5%) than those without surgery (29%)[22].

In locally advanced tumors that involve surrounding organs or tissues, an aggressive surgical intervention is technically feasible in selected patients and may offer appropriate disease control[23]. Besides, resection of locally advanced tumors with major blood vessel involvement and the necessity for vascular reconstruction can be beneficial[24]. Unfortunately local recurrence is frequent after these interventions, and surgery in most cases is an intervention offering long term palliation rather than cure[25]. Interestingly, a margin-positive resection in locally advanced PNETs seems to offer a similar overall survival compared to margin-negative resections[26]. Therefore, a resection of locally advanced PNETs might even be attempted when margin-positivity is expected; however, a pre-operative assessment of putative tumor biology is

the key to successful PNET surgery.

Metastatic and recurrent disease

Liver metastases are commonly observed in PNEN patients and are present in up to 60% at initial diagnosis[27]. At that point, only a small fraction of patients are technically and/or oncologically resectable[28]. However, the presence of both synchronous or metachronous liver metastases does not generally represent a contraindication to surgical treatment of PNEN patients[29,30]. It is still unclear when and whether the primary tumor should be resected in non resectable metastatic disease[31]. Concerning liver metastases, a significantly higher 5 year survival (72% *vs* 25%) and a longer median survival (96 mo *vs* 20 mo) has been observed in resected patients compared to non-resected ones[32]. A relevant oncological benefit can be achieved by palliative surgical debulking of more than 90% of liver metastases, as also advocated by the recent ENETS guidelines[7,33,34]. In the presence of bilobar hepatic PNEN metastasis, resections may be performed safely in two-stage procedures in selected patients[35]. In addition, in the palliative setting, surgical cytoreduction has proven more efficient than transarterial chemoembolisation alone[36]. Another more recent option for the treatment of disseminated liver metastases is the selective internal radiation therapy (SIRT) with yttrium-90 labeled glass microspheres[37]. This radioembolisation therapy has been shown to be especially effective for the treatment of liver metastases of colorectal and neuroendocrine tumors[38]. However, multi-disciplinary therapeutic approaches in specialized centers are frequently required to maximize tumor mass reduction. In particular, surgical resection can be complemented by other liver-directed therapies such as radiofrequency ablation[32] or transcatheter arterial (chemo)embolisation[6].

Recurrence is a frequent finding and therefore reoperation for metastatic disease is frequently needed and can result in excellent long term survival of up to 70% after 10 years[30]. For early detection of PNEN recurrence, gallium-68 DOTATATE PET-CT may be helpful[39]. PNENs with a KI-67 index of more than 5%, positive lymph nodes, and tumor size > 4 cm are associated with a significantly higher risk for recurrence[40,41]. These data demonstrate that aggressive surgical resection can improve survival even in metastatic and recurrent disease.

In selected individual cases liver transplantation may be a treatment option, but evidence is limited and the oncological outcome uncertain[42]. Rosneau and colleagues reported 1-, 5- and 10- years survival rates of 89%, 80% and 50% in a study involving 17 patients[43] which is not better than what can be achieved by aggressive surgical debulking[33]. Furthermore, considering the lack of organs, this indication is reserved for highly selected patients[7,44]. The UNOS/Eurotransplant waiting lists for liver transplantation stratify patients by disease severity using the (lab)MELD score based on laboratory parameters. For some diseases (*e.g.*, HCC or cystic fibrosis) this labMELD score does not sufficiently mir-

ror disease severity, therefore standard exceptions have been defined for which a matchMELD can be assigned. However, liver metastases of neuroendocrine tumors are not considered a standard exception according to current waiting list criteria of Eurotransplant/UNOS and therefore these patients do not qualify for a standard exception matchMELD. Nevertheless, in cases where the treating physicians believe that liver transplant might be a viable option, a non-standard exception matchMELD score can be applied for. These exceptional cases are then judged upon on an individual basis by an expert committee. Steve Jobs, former CEO of Apple Inc., is the most prominent of these very select cases and received a liver transplant for metachronous PNEN liver metastases at the Methodist University Hospital Transplant Institute in Memphis, Tennessee in 2009[45,46].

PNECs-high grade disease

Pancreatic neuroendocrine carcinomas with a high KI-67 index show an increased risk for recurrence and metastatic disease and survival is poor[40]. Therefore resection in patients with poorly differentiated PNECs with a high proliferation index should currently only be attempted when an R0 resections seems possible[7]. There is currently no role for cytoreductive surgery in these highly malignant cases. In advanced disease, targeted therapies (*e.g.*, VEGF and mTOR inhibitors) are increasingly acknowledged to be superior to conventional chemotherapy in case of poorly differentiated PNECs.

PROGNOSTIC CONSIDERATIONS

For all PNENs, the 5- and 10-year survival rates are about 65% and 45%, respectively[47]. Tumor grade plays a significant prognostic role since patients with high grade PNENs have a much worse 5-year survival of less than 30%[48]. Other positive prognostic factors include young age < 55 years and absence of distant metastases[48]. Of these three parameters, Bilimoria and colleagues developed a prognostic score predicting survival after surgical resection[48]. For this prognostic score, points from 0 to 3 are given for age (< 55 years = 0 points, 55-75 years = 1 point, > 75 = 2 points), differentiation (well/moderately differentiated = 0 points, poorly differentiated = 1 point), and metastases (none = 0 points, liver = 1 point, other distant = 3 points)[48]. A prognostic score from 1 to 3 can then be assigned where prognostic score 1 was defined as a total of 0 points, a prognostic score of 2 was defined as a total of 1-2 points and a prognostic score 3 was defined as > 2 points[48]. Using this scoring system - which can be easily applied to every patient as soon as histology is available - Bilimoria and colleagues were able to show that patients with a prognostic score 1 had a favorable 5 year survival of 76.7% compared to 50.9% for prognostic score 2 and 35.7% for prognostic score 3[48]. While these data have been generated retrospectively and a validation on an independent cohort is lacking, this tool may still be helpful in estimating patient's survival and may therefore assist in adjuvant treatment decisions.

CONCLUSION

PNENs are rare neoplasms of the pancreas with a disease course considerably different from PDAC. Aggressive and extensive surgery may be an option for many patients suffering from locally advanced and even metastatic disease. This may in select cases involve resection of multiple organs to achieve a significant reduction of the tumor mass. In patients with PNEN liver metastasis, debulking of > 90% of the macroscopically visible tumor mass - if technically feasible - seems to extend overall survival. However, current evidence stems from retrospective, non-randomized studies, but obvious ethical and feasibility considerations preclude realization of such a trial. In addition, exceptional heterogeneity of PNENs in terms of tumor biology renders thoughtful design of inclusion and exclusion criteria of such a trial impracticable.

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2014 ADVANCES IN PANCREATIC CANCER

Improving outcomes in pancreatic cancer: Key points in perioperative management

José M Álamo, Luis M Marín, Gonzalo Suarez, Carmen Bernal, Juan Serrano, Lydia Barrera, Miguel A Gómez, Jordi Muntané, Francisco J Padillo

José M Álamo, Miguel A Gómez, Jordi Muntané, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBEREH o Ciberehd), Instituto de Salud Carlos III, 28029 Madrid, Spain

Luis M Marín, Gonzalo Suarez, Carmen Bernal, Juan Serrano, Lydia Barrera, Francisco J Padillo, Department of General Surgery, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, 41013 Seville, Spain

Author contributions: Álamo JM, Marín LM, Suarez G, Bernal C, Serrano J and Barrera L substantially contributed to conception and design, acquisition of data, or analysis and interpretation of data; Gómez MA and Muntané J contributed to drafting the article or revising it critically for important intellectual content; Padillo FJ contributed to final approval of the version to be published.

Correspondence to: Francisco J Padillo, MD, PhD, Department of General Surgery, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Av. Manuel Siurot s/n, 41013 Seville, Spain. javierpadilloruiz@gmail.com

Telephone: +34-955- 923122   Fax: +34-955-923002
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Abstract

This review focused in the perioperative management of patients with pancreatic cancer in order to improve the outcome of the disease. We consider that the most controversial points in pancreatic cancer management are jaundice management, vascular resection and neo-adjuvant therapy. Preoperative biliary drainage is recommended only in patients with severe jaundice, as it can lead to infectious cholangitis, pancreatitis and delay in resection, which can lead to tumor progression. The development of a phase III clinical trial is mandatory to clarify the role of neo-adjuvant radiochemotherapy in pancreatic adenocarcinoma. Venous resection does not adversely affect postoperative mortality and morbidity, therefore, the need for venous resection should not be a contraindication to surgical resection in selected patients. The data on arterial resection alone, or combined with vascular resection at the time of pancreatectomy are more heterogeneous, thus, patient age and comorbidity should be evaluated before a decision on operability is made. In patients undergoing R0 resection, arterial resection can also be performed.

Core tip: The pancreatic cancer is one of the most virulent malignancies. The review is focused in the different perioperative management of the patients with pancreatic cancer in order to improve the outcome of the disease.

INTRODUCTION

Approximately 45000 people will develop exocrine pancreatic cancer in 2013 in the United States. A high percentage (85%) of diagnosed cases will die which shows the virulent nature of this malignancy[1]. Surgical resection offers the only chance of cure.

Unfortunately, the vast majority of patients are diagnosed with locally advanced unresectable or metastatic

disease. Up to 15%-20% of patients are eligible for initial resection[2]. Furthermore, even for those undergoing complete resection (R0) the prognosis is poor, because most of these patients will eventually relapse and die of their disease.

Reported five-year survival rates following pancreaticoduodenectomy for node-negative and node-positive disease are 25%-30% and 10%, respectively[3].

There are many interesting factors in the perioperative management of pancreatic cancer which could result in an improvement in the long-term outcome of this aggressive disease, such as intraoperative radiation therapy, standard or extended lymphadenectomy and adjuvant chemotherapy. However, we consider that the most controversial points nowadays are jaundice management, vascular resection and neo-adjuvant therapy.

PREOPERATIVE DRAINAGE IN JAUNDICED PATIENTS

The most frequent location of pancreatic cancer is the head of the pancreas; therefore, obstructive jaundice is a common presenting symptom. Pre-operative biliary drainage has been used to provisionally resolve the obstruction and may reverse the dysfunction resulting in obstruction of biliary flow. In recent years, this issue has been controversial. However, there is insufficient evidence on this therapeutic option. Several positive outcomes were observed after preoperative drainage in jaundiced patients: (1) higher postoperative morbi-mortality is associated with prolonged acute-phase response. More than 10 d of biliary tract obstruction was related to an increase in endotoxin levels, and a positive acute-phase response peak[4]. After biliary drainage a transitory improvement in these alterations was observed, although values remained high 1 wk post-drainage[5]; (2) malignant obstructive jaundice *per se* induces significant changes in food intake. Anorectic endocrine mediators, liver injury and biliary obstruction are related to protein-caloric malnutrition. This is a reversible situation. Nutritional markers improve after new bile flow into the duodenum[6]; (3) patients with biliary tract obstruction who require surgery often have protein calorie malnutrition, which is associated with increased peri-operative morbidity and mortality. Internal biliary drainage yields good results, and experimental studies have shown that it may improve nutritional status. The levels of pre-albumin and transferrin improved 10 d after internal biliary drainage for both benign and malignant obstruction[7,8] as nutritional alterations in patients with obstructive jaundice were determined by the intensity of the biliary obstruction[5]; (4) fluid administration expands the extracellular water compartment before drainage, but fails to improve renal function after drainage. Definitive improvement in endocrine and renal function requires the restoration of bile flow into the duodenum[9]; and (5) plasma levels of atrial natriuretic peptide increase due to obstruction of the biliary tree[10]. In these cases, this may reflect a subclinical myocardial dysfunction related to the severity of jaundice. There is a measurable improvement in cardiac function after internal biliary drainage[11].

The safety of routine pre-operative biliary drainage has not been established[12]. Pre-operative biliary drainage may increase the rate of serious adverse events, such as a significant increase on the rate of bile cultures positive for bacteria and significantly increase the probability of wound infection. In addition, bile cultures positive for bacteria seem to adversely impact mortality and morbidity after surgery in jaundiced patients[13]. In a large multicenter randomized trial comparing early surgery *vs* preoperative biliary drainage followed by surgery in patients with cancer of the pancreas head, the rates of serious complications were 39% (37 of 96 patients) in the early surgery group and 74% (75 of 106 patients) in the patients submitted to preoperative biliary drainage ($P \leqslant 0.001$)[14]. A follow-up report from the same trial showed that there was a significant delay in time to surgery (1 wk *vs* 5 wk), but no influence on survival rate[15]. While there was an increase in overall infectious complications following surgery in the stented group, the detrimental effects of pre-operative biliary stenting were likely limited to those with subsequent bacterial colonization of the biliary tree due to stent placement[16].

The rapid and direct scheduling for surgery may limit the number of interventions and thus decrease costs and potential procedure-related complications. Siddiqui *et al*[17] observed immediate complications such as post-operative endoscopic retrograde cholangiopancreatography pancreatitis ($n = 14$), stent migration ($n = 3$), and duodenal perforation ($n = 3$), as well as long-term complications included stent migration ($n = 9$) and hepatic abscess ($n = 1$). Fourteen patients (5.8%) experienced stent occlusion at an average of 6.6 mo (range 1 to 20 mo) after surgery. A total of 144 out of 174 patients (83%) deemed to have resectable cancer at the time of diagnosis subsequently underwent curative surgery. Due to disease progression or the discovery of metastasis after neo-adjuvant therapy, only 22 of 67 patients (33%) with borderline-resectable cancer underwent curative surgery.

The pre-operative placement of biliary stents in patients undergoing pancreatoduodenectomy significantly increases blood loss, with non-significant increases in operative time and peri-operative fluid resuscitation. In this cohort, these intra-operative considerations do not translate into increased peri-operative morbidity and mortality, with the data overall showing negligible differences in improved outcomes in stented patients. Consequently, pre-operative biliary stents may complicate intra-operative surgical management[18].

NEO-ADJUVANT THERAPY IN PANCREATIC CANCER

The low rate of resectability and the poor long-term outcomes following pancreatoduodenectomy have led to the investigation of pre-operative chemo-radiation therapy or a combination of pre-operative and post-operative

Table 1 Methods data of the three published meta-analyses on neo-adjuvant therapy in pancreatic carcinoma *n* (%)

	Year	*n*	Type study	Mean age (yr)		Chemotherapy Agents regimen		Radiotherapy Dose (Gy) IORT		
Gillen *et al*[2]	80-09	111	78P-33R	62.5	107 (96.4)	5FU > GEM > Tax > Others	44S + 48C	104 (93.7)	24-63	13 (12.5)
Assifi *et al*[35]	93-10	14	14P-0R	N/P	14 (100)	GEM > 5FU	3S + 11C	12 (85)	30-50	0 (0)
Andriulli *et al*[36]	97-08	20	20P-0R	63.0	20 (100)	GEM > Cis	13S + 7C	17 (85)	30-40	N/P

P: Prospective; R: Retrospective; 5FU: 5-fluor-uracil; GEM: Gemcitabine; Cis: Cisplatine; Tax: Taxanes; S: Single; C: Combined; IORT: Intraoperative radiotherapy; N/P: Not provided.

Table 2 Results of the three published meta-analyses on neo-adjuvant therapy in pancreatic carcinoma in terms of safety (postoperative morbidity and toxicity) and efficacy (response and resection)

		Response (%)			Resection (%)				
	Toxicity (%)	Complete	Partial	Progression	Resected	R0	Mono	Combined	Postoperative morbidity (%)
Gillen *et al*[2]	01:26.3	3.6	30.6	20.9	73.6	60.4	80.9	66.2	26.7
	02:31.3	4.8	30.2	20.8	33.2	26.2	27.3	33.0	39.1
Assifi *et al*[35]	1:37	0.8	9.5	17.0	65.8	55.9	N/P	N/P	N/P
	02:46.2	4.0	31.8	21.8	31.6	19.6			N/P
Andriulli *et al*[36]	1:29	12		15.0	81.2	66.4	N/P	N/P	N/P
	2:33	27		32.0	26.4	16.0			N/P

1: Group of patients with potentially resectable pancreatic adenocarcinoma; 2: Group of patients with borderline resectable pancreatic adenocarcinoma. Toxicity: Only grade 3 and 4; Resection R0: Complete resection of the tumor; Resection Mono: Single chemotherapy drug; Resection Combined: Combined chemotherapy drugs; N/P: Not provided.

Table 3 Results of the three published meta-analyses on neo-adjuvant therapy in pancreatic carcinoma in terms of survival and mortality

	Mean Survival (mo)	Mortality (%)	Estimated survival (%) 1-yr	2-yr
Gillen *et al*[2]	01:23.3	3.9	77.9	47.4
	02:20.5	7.1	79.8	50.1
Assifi *et al*[35]	01:15.1	N/P	N/P	N/P
	02:11.2			
Andriulli *et al*[36]	01:18.8	N/P	91.7	86.3
	2:14		67.2	54.2

Referred only after surgical resection. 1: Group of patients with potentially resectable pancreatic adenocarcinoma; 2: Group of patients with borderline resectable pancreatic adenocarcinoma. N/P: Not provided.

therapies[19]. In this context, neo-adjuvant therapy is defined as any pre-operative therapy aiming to convert un-resectable to resectable tumors and/or to increase microscopic complete tumor resection rates[20]. Given this situation, the rationale for neo-adjuvant therapy in pancreatic cancer are as follows[21]: (1) the main objective is down-staging of the tumor to increase the probability of survival after an R0 resection; (2) a certain percentage of potentially un-resectable tumors may be down-staged to enable surgical resection; (3) radiation therapy is more effective on well-oxygenated cells that have not been de-vascularized by surgery; (4) pre-operative treatment may prevent implantation and dissemination of tumor cells at laparotomy; (5) patients with metastatic disease on restaging after neo-adjuvant therapy will not be subjected to unnecessary laparotomy; and (6) delayed post-operative

recovery will not affect the delivery of neo-adjuvant therapy.

Candidates for neo-adjuvant therapy are those with radiographically resectable and biopsy-proven pancreatic adenocarcinoma[22]. Numerous phase II trials have been performed with encouraging results[23-25]. Although median survival durations from some uncontrolled trials showed that neo-adjuvant therapy compared favorably with modern adjuvant therapy approaches[24,26,27], whether pre-operative therapy is better than post-operative therapy is uncertain. No phase III trial comparing neo-adjuvant and post-operative adjuvant therapy has been performed, however, there are many retrospective comparisons using the borderline resectable pancreatic cancer criteria[28] which favor neo-adjuvant therapy for these cancers that almost certainly would have had a positive resection margin if surgery were performed first[29-31]. Moreover, such retrospective studies may have sample selection bias[32].

In this review we distinguish the results of neo-adjuvant therapy between patients with potentially resectable (Group 1) and borderline resectable pancreatic adenocarcinoma (Group 2). In fact, this is one of the main limitations of different meta-analyses, as the criteria for considering borderline carcinoma are heterogenous. The expert consensus statement was published in 2009[33]. The conclusions of the three published meta-analyses (level of evidence 1+ of the SIGN related to neo-adjuvant therapy in pancreatic cancer are shown in Tables 1, 2 and 3[34].

The methods data of the three published meta-analyses on neo-adjuvant therapy in pancreatic carcinoma (Table 1) are different. Gillen *et al*[2] included retrospective

and prospective phase Ⅰ-Ⅱ trials, as well as cohort studies and case series during an interval of 29 years (from 1980 to 2009) with an important variety of neo-adjuvant regimens.

The authors consider that the heterogeneity of the data is a limiting factor for extrapolation of the results. However, this first meta-analysis concluded that patients with locally advanced/un-resectable tumors should be included in neo-adjuvant protocols and subsequently be re-evaluated for resection, which is possible in a relevant number of patients. Moreover, in the group of patients with resectable tumors, resection and survival rates after neo-adjuvant therapy were similar to those observed in primary resected tumors treated with adjuvant therapy. Thus, in this group of patients, the current data do not demonstrate an obvious advantage of neo-adjuvant therapy. The study designs provided by Assifi *et al*[35] and Andriulli *et al*[36] are less heterogeneous. The data collection was limited only to prospective phase Ⅱ trials investigating the effects of neo-adjuvant therapy on patients with pancreatic cancer during a similar time period. The last study included patients receiving gemcitabine alone or in combination with other drugs and/or radiotherapy. The problem of heterogeneity found in all meta-analyses was handled satisfactorily using the random effects model and a $P < 0.10$ in the Cochran Q test in the case of Assifi *et al*[35]. Despite a rigorous selection of studies, Andriulli *et al*[36] found significant heterogeneity which may indicate that the evidence was biased, confounded or inconsistent. Two factors which could, at least partly, explain the heterogeneity were identified. First, the patients' initial disease stage (resectable *vs* un-resectable) and, second, the study design. We think that one of the main limitations of the meta-analyses was the definition of unresectability and borderline resectability. These terms were not consistent between the studies, or clearly described in the manuscripts. Although the definitions have recently undergone standardization[33], the majority of studies analyzed preceded the adaption of such definitions or they were not utilized by the authors.

A recent meta-analysis of prospective studies published by Festa *et al*[37] involving patients who received chemotherapy with or without radiotherapy which was given before surgery to patients with borderline resectable cancer, estimated that the surgically explored and resection rate was higher in patients who received preoperative treatment with gemcitabine. Promising results in retrospective studies have been reported with neo-adjuvant FOLFIRINOX in borderline resectable pancreatic adenocarcinoma followed by radiation[25]. We have assessed the results of the meta-analyses in terms of safety (toxicity of the neo-adjuvant regimen and postoperative morbidity), efficacy (response and resection rate), survival and mortality (Tables 2 and 3). Toxicity data were not available in all the studies revised in the three meta-analyses.

However, these studies agree on the increasing incidence of grade 3-4 toxicity with the combined therapy (two or more chemotherapeutic agents or radiotherapy). In spite of the high estimated heterogeneity of these results, toxicity was higher in the group of patients who were borderline resectable than in those with potentially resectable pancreatic adenocarcinoma[2,35,36].

Postoperative morbidity was only reported by Gillen *et al*[2], and the results are comparable to others series[38,39]. In a systematic review reported by Laurence *et al*[40], neo-adjuvant chemoradiotherapy was not associated with a statistically significant increase in the rate of pancreatic fistula formation or total complications. One of the most important aspects of this review was the response and resection rate after neo-adjuvant therapy. A 30% response rate (complete and partial) in borderline resectable patients provides marginal support for the benefit of preoperative therapy.

The median survival of patients with locally advanced unresectable pancreatic cancer is approximately 10 to 12 mo. Interest in applying the principles of neo-adjuvant or induction therapy to such patients is due to their poor prognosis and the potential for longer term survival if the disease can be resected. Both Gillen *et al*[2] and Andriulli *et al*[36] calculated that the 1-year and 2-year estimated survival were 75% and 50%, respectively.

However, these data must be interpreted cautiously given the heterogeneous nature of this group of patients and their treatments. The influence of preoperative therapy on patient survival remains uncertain. Whether the improved median survival times in resected patients can be ascribed to the chemoradiotherapy administered before surgery or to a better selection of patients with non-progressive disease during the interval from diagnosis to completion of chemoradiotherapy and restaging remains to be addressed in a properly designed randomized trial[36].

It is probable that if pancreatic cancer can be completely resected, the best option is still surgical resection; neo-adjuvant therapy (chemotherapy or chemoradiotherapy) should be given in those patients with doubtful R0 resection, mostly locally advanced tumors, although this definition is not clearly defined.

VASCULAR RESECTION IN PANCREATODUODENECTOMY

The objective of vascular resection in case of vascular tumor invasion in pancreatic cancer is a potentially curative resection. Metastases must be the reference to performance a venous or arterial resection, so we must not practice it if there would be metastases in peritoneum or other organs. Venous invasion usually affects the superior mesenteric vein (SMV) or portal vein (PV), while hepatic artery (HA) the superior mesenteric artery (SMA) are the most affected arteries in pancreatic cancer. The indications and outcomes of vascular resections in pancreatic cancer are still in continuous study.

The purpose of vascular resection is, obviously, to increase the possibility of a curative R0 resection, because

a complete resection is the most important prognostic factor that influences long time survival. This is the reason why obtaining tumor-free resection margins must be the most important objective during vascular resection in pancreatitis ic cancer. In our experience, we have operated on 22 patients with pancreatic cancer including vascular resection:5 with arterial and 17 with venous resection (2005-2013). The mortality associated with the procedure was 36.4% (8 patients), and 6 surviving patients showed tumor recurrence (27.3%). The 5-year survival rate was 36.4% (range 1-96 mo, median 54 mo).

Arterial resection

The narrowing or vessel encasement of SMA, HA or celiac trunk (CT) observed on CT scan[41] or intraoperatively is usually due to a locally advanced tumor, but sometimes, this narrowing is secondary to a peri-tumour fibrosis, and this fact is most of times very difficult to define before or during surgical procedure. Furthermore, if we are sure that this arterial invasion indicates unresectability is in order to technical aspects and prognosis, highly debatable.

There are some doubts about arterial infiltration: (1) is arterial invasion result of an advanced carcinoma or is because of cancer location, near of these important vessels? (2) Is it supposed the finding of arterial infiltration the patient is in stage IV situation? (3) Do arterial resections influence in complications and mortality after pancreatic resection? Several articles show similar long-term survival in patients with arterial invasion compared with patients without vascular invasion. The fact that microscopy showed that vascular tumor invasion is an adverse factor has been changed by these studies[42-44]. This could be explained because the most important factor in survival in patients with pancreatic carcinoma is the presence of metastases in peritoneum or other organs. Yekebas *et al*[45] showed that arterial resection can be a safely procedure in cases with secure vascular invasion, being morbidity and mortality rates comparable to pancreatectomies without arterial resection. In this article, vascular resection did not influence in survival after surgery. When potentially curative pancreatectomy is performed, 2- and 5-year survival rates in patients with vascular invasion are 35% and 15%, respectively, the same rates that patients without arterial invasion. The median survival after pancreatectomy with arterial resection is 6 and 39 mo, much longer than pancreatic cancer treated with chemotherapy or palliative surgery. Although tumor arterial invasion of more than 180º is considered the most important criterion for unresectable cancer in persons with pancreatic tumors according to updated guidelines[46], there is still insufficient data to assert this fact.

The advances in pancreatic surgery together with the poor survival of patients who do not undergo surgical resection, have led to a debate regarding the importance of arterial resection in patients without distant metastasis. There are some studies on pancreatectomy with arterial resection in small series of patients. These articles showed that overall survival in patients with arterial re-

section is significantly worse when compared with operated patients without arterial resection. Vascular invasion should be considered an indicator of aggressive tumor biology, and it seems to be worse arterial than venous invasion: when simultaneous venous and arterial resection was performed in some studies, patients with arterial had a higher risk of R1 resection and more presence of affected nodes, so survival was reduced[47-49].

In the meta-analysis published by Mollberg *et al*[50] a significantly better survival was observed in patients with arterial resection compared with patients without tumor resection. The results of these analyses should be interpreted very cautiously, as it was an uncontrolled study: patients without resection with more advanced tumors had a worse prognosis compared to patients who underwent pancreatic and arterial resection. This meta-analysis found that patients with arterial resection had more postoperative complications and a worse long-term survival. The authors concluded that the need for arterial resection should be a contraindication to resectability. However, the survival benefit offered by pancreatectomy with arterial resection compared to palliative therapy without tumor resection could justify arterial resection in highly selected patients, only if performed at specialized institutions.

Bachellier *et al*[51] showed that pancreatic resection with arterial resection for locally advanced pancreatic cancer can be performed safely with survival rates similar to patients with locally advanced pancreatic adenocarcinoma without arterial resection (survival rates of 20% at 5 years). This study showed that perineural invasion, number of resected lymph nodes (< 15 *vs* > 15), and arterial wall invasion were independent prognostic factors for overall survival. The authors recommended the following: (1) radiological arterial invasion should not be considered a contraindication to pancreatic resection if the patient undergoes R0 resection; (2) the specificity of CT scanning to predict histological arterial wall invasion is still low; (3) in the case of radiological arterial invasion the patient should be a candidate for neo-adjuvant treatment; (4) after neo-adjuvant therapy in the absence of cancer progression an exploratory laparotomy should be performed to explore the resectability of the tumor; (5) arterial resection should be performed if the patient is undergoing R0 resection; and (6) pancreatic resection with arterial resection should be performed in specialized centers.

Bockhorn *et al*[52] reported a study of eighteen patients who required reconstruction of the HA, eight CT and three SMA. Fifteen patients also required resection of PV. Complications and mortality were significantly higher in patients with arterial resection than in patients without arterial resection ($P = 0.031$ and $P = 0.037$, respectively). Venous resection was an independent factor for morbidity ($P < 0.001$). Median overall survival was the same for both groups (14.0 *vs* 15.8 mo; $P = 0.152$). This article concluded than, in selected patients, overall survival following arterial resection was similar to standard resection and better than palliative treatment.

In conclusion, due to the doubtful data available, the operative and oncological results of these patients should be documented in centralized patient registries in prospective studies.

Venous resection

PV and SMV invasion is due to the location of the tumor, because this venous trunk is in the anatomic origin of pancreatic cancer. For a long time, venous invasion was regarded a contraindication to resection in pancreatic cancer. Today, there is controversy regarding arterial resection and whether pancreatic carcinoma with involvement of the PV/SMV should be resected.

The first resection and reconstruction of the PV/SMV during pancreatectomy were reported by Moore *et al*[53] (1951) and by Asada *et al*[54] (1963). In 1973, Fortner[55] proposed "regional pancreatectomy" which involved a systematic resection PV/SMV vessels and peripancreatic nodal and adipose tissue clearance, to increase the long-term survival rate. This procedure showed no survival benefit and was associated with high morbidity[56,57]; thus, most authors regard tumor invasion of the PV/SMV as a contraindication to curative pancreatic surgery.

However, several reports have confirmed that resection of the PV/SMV can be performed with acceptable mortality, complications and survival rates, comparable to those observed in pancreatic surgery without venous resection[43,44,58,59]. On the other hand, some author have reported poor survival results after this surgical procedure[60]. In general, the current opinion confirms the safety and feasibility of this surgical techniques, with mortality rates about 0 to 7.7%, which are similar to accepted mortality for pancreatectomy without venous resection reported in some studies[61-63]. Also, morbidity rates are similar to pancreatic resections performed without PV/SMV resections (16.7% to 54%)[64,65]. Reported 5-year survival rate in patients with venous resections is not different those without PV/SMV reconstruction (9%-18%)[66].

Many studies uphold PV/SMV resection during pancreatoduodenectomy, although some studies report a low 5-year survival rate because a venous infiltration leads to a more probability of nodal spread[67]. In a retrospective review of two prospective registers with 593 consecutive pancreatic resections for pancreatic cancer reported by Martin *et al*[68], 36 patients (18 men and 18 women, aged 42-82 years) (6.1%) underwent vascular resection at the time of pancreatectomy. Among them, 31 (88%) needed PV/SMV resection, 3 (8%) both arterial and venous resection; and 2 (6%) only arterial resection. The 90-d mortality was 0% and morbidity was 35%. In control group rates were 2% and 39% respectively ($P = 0.034$). Median survival was 18 mo in the venous or arterial resection group, and 19 mo in the control group.

The current literature suggests that PV/SMV resection while pancreatic resections, is a safe and feasible surgical technique, but this procedure must be made only in experienced centers with acceptable morbidity and mortality rates. Complication rates are similar to observed for pancreatic resections without venous reconstruction. Only venous resections can make R0 pancreatectomies in some cancer, and this is, today, the only curative therapy in these patients.

In conclusion, pancreatectomy combined with venous resection should always be considered in cases of suspected tumor infiltration of PV/SMV to obtain good resection margins, in the absence of distant metastasis. R0 resection continues to be the ultimate goal for patients with pancreatic carcinoma, because this is the most important technique in improving survival, thus, venous involvement should not contraindicate pancreatic resection, especially when R0 margins are possible and when reasonable reconstructions can be performed.

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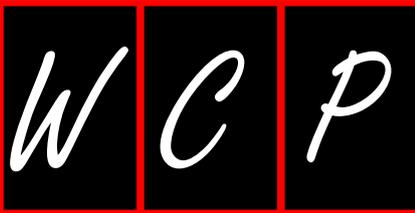
Laparoscopic resection of pancreatic adenocarcinoma: Dream or reality?

Blaire Anderson, Shahzeer Karmali

Blaire Anderson, Shahzeer Karmali, Department of Surgery, University of Alberta, Edmonton, Alberta T6G 2B7, Canada
Shahzeer Karmali, Center for the Advancement of Minimally Invasive Surgery, Royal Alexandra Hospital, Edmonton, Alberta T5H 3V9, Canada
Author contributions: Anderson B reviewed the literature and wrote the paper; Karmali S supervised the review.
Correspondence to: Shahzeer Karmali, BSc, MD, MPH, Assistant Professor, Surgical Director, Weight Wise Bariatric Surgery, Minimally Invasive Gastrointestinal and Bariatric Surgery, Center for the Advancement of Minimally Invasive Surgery, Royal Alexandra Hospital, Room 405 Community Services Center, 10240 Kingsway, Edmonton, Alberta T5H 3V9, Canada. shahzeer@ualberta.ca
Telephone: +1-780-7356650   Fax: +1-780-7356652
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Abstract

Laparoscopic pancreatic surgery is in its infancy despite initial procedures reported two decades ago. Both laparoscopic distal pancreatectomy (LDP) and laparoscopic pancreaticoduodenectomy (LPD) can be performed competently; however when minimally invasive surgical (MIS) approaches are implemented the indication is often benign or low-grade malignant pathologies. Nonetheless, LDP and LPD afford improved perioperative outcomes, similar to those observed when MIS is utilized for other purposes. This includes decreased blood loss, shorter length of hospital stay, reduced post-operative pain, and expedited time to functional recovery. What then is its role for resection of pancreatic adenocarcinoma? The biology of this aggressive cancer and the inherent challenge of pancreatic surgery have slowed MIS progress in this field. In general, the overall quality of evidence is low with a lack of randomized control trials, a preponderance of uncontrolled series, short follow-up intervals, and small sample sizes in the studies available. Available evidence compiles heterogeneous pathologic diagnoses and is limited by case-by-case follow-up, which makes extrapolation of results difficult. Nonetheless, short-term surrogate markers of oncologic success, such as margin status and lymph node harvest, are comparable to open procedures. Unfortunately disease recurrence and long-term survival data are lacking. In this review we explore the evidence available regarding laparoscopic resection of pancreatic adenocarcinoma, a promising approach for future widespread application.

Core tip: Laparoscopic pancreatic surgery is in its infancy despite initial procedures reported two decades ago. Both laparoscopic distal pancreatectomy and laparoscopic pancreaticoduodenectomy can be performed competently with improved perioperative outcomes, similar to those observed when minimally invasive surgical (MIS) is utilized for other purposes. However, when MIS approaches are implemented the indication is often benign or low-grade malignant pathologies. In this review we explore the evidence available regarding laparoscopic resection of pancreatic adenocarcinoma, a promising approach for future widespread application.

INTRODUCTION

In 2013 an estimated 45220 new cases of pancreatic

cancer will be diagnosed in the United States resulting in approximately 28460 deaths[1]. Pancreatic adenocarcinoma is one of the most aggressive malignant tumors. Overall, prognosis is dismal with recent 5-year survival estimates merely 6%[1]. Oncologic surgical resection is the only potentially curative treatment[2,3]. Unfortunately only 15%-20% of patients have resectable disease at presentation[4]. This is secondary to rapid local spread, invasion of critical surrounding structures, and early distant metastases. Surgical options are as follows; pancreatic body and tail tumors can be resected by subtotal or distal pancreatectomy (DP), while pancreatic head tumors are amendable to pancreaticoduodenectomy (PD).

LAPAROSCOPIC PANCREATIC SURGERY

Laparoscopic pancreatic surgery has been slow to evolve, primarily reserved for staging and palliation[5-9] despite the performance of initial minimally invasive surgical (MIS) pancreatic procedures two decades ago[10-12]. This delay can likely be attributed to multiple factors, including the retroperitoneal location of the pancreas, anatomic complexity of surrounding structures, friable nature of the gland, and propensity for post-operative complications. To date laparoscopic procedures currently executed include: (1) diagnostic laparoscopy with or without biopsy; (2) palliative interventions including gastro- and hepaticojejunostomy; (3) DP with or without splenectomy; (4) PD; (5) tumor enucleation; and (6) central pancreatectomy. Feasibility is well established[13-16]; however by no means are MIS techniques mainstream and when utilized reserved for benign and low-grade malignant tumors.

LAPAROSCOPIC SURGERY FOR PANCREATIC ADENOCARCINOMA

The results of the Clinical Outcomes of Surgical Therapy trial have promoted the implementation of MIS techniques in the treatment of colon cancer with equivalent recurrence and overall survival rates[17]. As well, further studies have demonstrated oncologic outcomes in MIS resection of other abdominal malignancies to be equivalent to open techniques[18-22]. MIS approaches offer the added benefits of decreased blood loss, shorter length of hospital stay, reduced post-operative pain, and expedited time to functional recovery. These improved perioperative outcomes are of paramount importance in regards to pancreatic adenocarcinoma, where improved recovery can mean earlier instigation of adjuvant treatment. Skepticism persists surrounding oncologically sound surgical resection, crucial in any malignancy. Additionally, due to tumor biology and aggressiveness of disease process pancreatic resections for adenocarcinoma are not commonly performed making it difficult to overcome the associated learning curve. Long-term survival data is scarce leaving evidence reliant on short-term surrogate markers including margin status and lymph node retrieval. In general, lack of quality data has hindered MIS progress for

pancreatic malignancies.

LAPAROSCOPIC DISTAL PANCREATECTOMY

Laparoscopic approaches to DP improve visualization of the retroperitoneal pancreas and minimize incisional trauma. This procedure is amendable to MIS techniques, as it requires limited dissection and no anastamoses. However, pancreatic stump management and the prevention of fistulae prove trying. Variations exist in the utilizations of hand-access approaches, which provide the benefit of improved tactile feedback, and splenic preservation, which has been reported to reduce postoperative and overall infectious complications[23]. Overall, cosmesis, reduced blood loss, shorter length of hospital stay, less post-operative pain, and expedited time to functional recovery results from laparoscopic distal pancreatectomy (LDP)[14,24-33]. Additionally, in a Canadian study, Fox et al[34] demonstrated equal surgical costs, decreased length of stay, and decreased overall costs ($10842 vs $13656 for LDP and ODP respectively). Long-term oncologic outcomes have yet to be reported; however R0 resection and lymph node harvest are comparable to open resections (Table 1)[35-47].

Pancreatic fistulae

In several large case control series morbidity has proven to be comparable to open approaches (Tables 1 and 2). The potentially devastating nature of complications associated with pancreatic fistula has led to great debate regarding the optimal management of the pancreatic stump[48]. Techniques currently utilized include staple closure with or without staple line reinforcement, electrocautery, ultrasound coagulation, radiofrequency, omental patch, fibrin glue, enteric anastomosis, occtreotide administration, or a combination[49-55]. There has yet to be a consensus on the best method for stump management, even in the literature for open procedures[55]. Kooby et al[45] reported fistula rates to be similar between LDP and ODP (26% vs 32%, P = 0.28). In this study linear stapler was utilized for pancreatic transaction. Topical sealants and perioperative octreotide were added at the discretion of the treating surgeon. With MIS approaches providing comparable results in terms of pancreatic fistula rates, this should not deter laparoscopic advancement.

Splenic preservation

MIS approaches lead to greater splenic preservation[26,45,56]; however, in the case of malignancy, proximity of tumor to splenic vasculature often makes preservation difficult while achieving oncologic resection. The Warshaw technique, in which splenic vessels are resected en bloc, leaves the spleen to survive on the short gastric and left gastroepiploic vessels; providing an alternative more oncologically appropriate approach[57]. Complications associated with this method include gastric variceal hemorrhage and splenic infarction. Ferrone et al[58] in a retrospective

Table 1	Published comparisons of laparoscopic distal pancreatectomy and open distal pancreatectomy with at least five cases of adenocarcinoma																

Study	Cases		Malignant pathology		Mean node harvest		R0 margins		Mean blood loss (mL)		Mean length of stay (d)		Pancreatic fistula rate		Mortality		Overall morbidity	
	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP	LDP	ODP
Rehman *et al*[35]	8	14	100%	100%	16[1]	14[1]	88%	86%	306	650	8	12	25%	21%	0%	0%	NR	NR
Mehta *et al*[36]	30	30	23%	23	8	14	NR	NR	294	726	9	13	17%	13%	0%	3%	50%	43%
Limongelli *et al*[37]	16	29	36%	45%	NR	NR	94%	93%	160	365	6	9	18%	20%	0%	3%	25%	41%
Abu Hilal *et al*[38]	35	16	19%	11%	NR	NR	75%	67%	200	394	7	11	29%	44%	0%	6%	40%	69%
DiNorcia *et al*[39]	71	168	13%	39%	6	8	97%	87%	150	900	5	6	11%	14%	0%	1%	28%	44%
Vijan *et al*[40]	100	100	23%	23%	NR	NR	0%	0	171	519	6	9	17%	17%	3%	1%	34%	29%
Kooby *et al*[41]	23	189	100%	100%	14	12	73%	74%	422	790	7	11	NR	NR	0%	1%	NR	NR
Jayaraman *et al*[42]	107	236	17%	47%	6	7	97%	96%	175	300	5	6	15%	13%	0%	1%	26%	33%
Baker *et al*[43]	27	85	29%	30%	5	9	NR	NR	219	612	4	8	22%	14%	0%	2%	37%	35%
Finan *et al*[44]	44	98	25%	42%	NR	NR	0%	0%	157	719	6	9	50%	46%	0%	5%	NR	NR
Kooby *et al*[45]	142	200	36%	49%	NR	NR	92%	93%	357	588	6	9	11%	18%	0%	1%	40%	57%
Eom *et al*[46]	31	62	10%	7%	NR	NR	NR	NR	NR	NR	12	14	10%	7%	0%	0%	36%	24%
Velanovich[47]	15	15	20%	32%	NR	NR	NR	NR	NR	NR	5	8	13%	13%	0%	0%	20%	27%

[1]Median. NR: Not reported; LDP: Laparoscopic distal pancreatectomy; ODP: Open distal pancreatectomy.

Table 2	Definitions of post-operative pancreatic fistulae
Study	**Definition of post-operative pancreatic fistula**
Rehman *et al*[35]	ISGPF definition
Mehta *et al*[36]	ISGPF (grade B and C)
Limongelli *et al*[37]	ISGPF definition
Abu Hilal *et al*[38]	ISGPF definition
DiNorcia *et al*[39]	ISGPF definition
Vijan *et al*[40]	ISGPF definition
Kooby *et al*[41]	NR
Jayaraman *et al*[42]	ISGPF definition
Baker *et al*[43]	ISGPF definition
Finan *et al*[44]	ISGPF definition
Kooby *et al*[45]	ISGPF definition
Eom *et al*[46]	Drainage > 30 mL with amylase > 600 U/dL
Velanovich[47]	Amylase-rich fluid after POD 3

ISGPF: International Study Group on Pancreatic Fistula; NR: Not reported; POD: Post-operative day.

review of 721 patients who underwent open distal pancreatectomy, 158 of who had a Warshaw spleen preserving distal pancreatectomy, reported evidence of perigastric varices in 25% of patients on follow-up computed tomography scan. Perhaps a paraphysiologic finding, as no clinically relevant adverse events were observed at up to 21 years follow-up. Re-operation, splenectomy, for splenic infarction was required in only 3 cases. From this, it was postulated that splenic preservation using the Warshaw technique should be considered in patients undergoing distal pancreatectomy for nonmalignant pathologies. Only 6% of this study population had pancreatic adenocarcinoma leaving reservations in the extrapolation of results to this subgroup. Similar findings have been published in laparoscopic series[25,59-63]. Jean-Philippe Adam *et al*[64] reviewed a cohort of 154 patients who underwent LDP with splenic preservation. Again, the indication for operation was benign or low-grade malignant tumors. Overall morbidity, pancreatic fistula, and reoperation were similar regardless of technique. Splenic preservation was less successful with the Warshaw technique compared with splenic vessel preservation, 84.7% *vs* 96.4% respectively (*P* = 0.03). Nine patients in the Warshaw group presented to hospital with symptomatic post-operative splenic infarction, necessitating splenectomy in four. Greater splenic preservation without compromised oncologic resection is favorable and achievable with laparoscopic approaches.

Oncologic outcomes

Potential for cure in this disease process is approached with R0 resection, therefore margin status is of paramount importance[65]. Another short-term surrogate measure of oncologic outcome is lymph node harvest. Venkat's[24] recent systematic review and meta-analysis reported no significant difference between R0 resection in LDP, 95.5%, and ODP, 91.2% (*P* = 0.27). Meta-analysis was not completed for lymph node harvest due to inadequately reported data. A retrospective cohort study by Jayaraman *et al*[42] compared 343 patients. The laparoscopic group had fewer cancer cases (17% *vs* 47%, *P* < 0.0001); however oncologic outcomes including R0 negative margins (97% *vs* 96%, *P* = 0.76) and lymph node harvest (6 *vs* 7, *P* = 0.53) were equivalent. Subsequently, Kooby *et al*[41] in a multicenter analysis of LDP (11%) *vs* ODP (89%) for adenocarcinoma, which included 212 patients, published similar results for R0 resection (27% *vs* 26% positive margins, *P* = 0.98) and lymph node harvest (12.5 ± 8.5 *vs* 13.8 ± 8.4 nodes, *P* = 0.47). DiNorcia *et al*[39] retrospective cohort study of 360 patients reported lymph node yield (6 LDP *vs* 8 ODP, *P* = 0.29); however more successful R0 resection in the LDP group with 2.8% positive margins compared to 13% in the ODP group (*P* = 0.01). Song *et al*[62] presented results of a 359 patients case series, 6.7% with adenocarcinoma. They achieved a 91% R0 resection and mean lymph node harvest of 10.3 ± 8.6 with overall survival rates of 85.2% at up to 2 years follow-up. Contrarily, Baker *et al*[43] in a retrospective cohort study of 112 patients reported a "less robust lymphadenectomy" with 5 compared to

Table 3 Published series on laparoscopic and robotic pancreaticoduodenectomy with at least five cases of adenocarcinoma (variables reported for entire series, not just malignant cases)										
Study	Cases	Malignant pathology	Mean node harvest (range)	R0 margin	Mean blood loss (mL)	Mean length of stay (d)	Pancreatic fistula rate	Mortality	Overall morbidity	Mean operative time (min)
[2]Bao *et al*[77]	28	4 AC 10 PDAC	15 (8-32)[1]	63%	100[1]	7[1]	21%	7%	NR	431[1]
Kim *et al*[78]	100	4 AMP 7 PDAC	13 (7-34)	100%	NR	14	6%	1%	25%	487
Asbun *et al*[76]	53	8 AMP 22 PDAC	23 (SD 10)	95%	195	8	17%	6%	22%	608
[2]Chalikonda *et al*[79]	30	14 adeno-carcinoma	13 (1-37)	100%	486	10	7%	3%	30%	476
[2]Lai *et al*[80]	20	5 AC 7 PC	10 (SD 60)	73%	247	14	35%	0%	50%	492
[2]Zeh *et al*[81]	50	9 AMP 14 PDAC	17 (5-37)	89%	350[1]	10	20%	2%	30%[3]	568[1]
Suzuki *et al*[72]	6	4 AMP 1 PDAC	18 (16-27)	100%	471[1]	23[1]	33%	0%	33%	581[1]
Ammori *et al*[15]	7	2 AMP 5 PDAC	20.8 (11-32)	NR	350	11	14%	0%	29%	628
[2]Giulianotti *et al*[82]	60	15 AMP 27 PDAC	18 (5-45)	92%	394	22	31%	14%	31%	421
Kendrick *et al*[83]	62	8 AMP 31 PDAC	15 (6-31)	89%	240	7	18%	2%	42%	368[1]
Cho *et al*[66]	15	1 AMP 4 PDAC	19 (NR)	100%	445	16	13%	0%	27%	338
Palanivelu *et al*[84]	75	29 AMP 33 PDAC	14 (8-22)	97%	74	8	7%	1%	27%	357
Pugliese *et al*[67]	19	4 AMP 6 PDAC	12 (4-22)	100%	180	18	23%	0%	37%	461
Dulucq *et al*[75]	25	4 AMP 11 PDAC	18 (NR)	100%	107	16	5%	5%	32%	287
Staudacher *et al*[69]	7	1 PDAC	26 (16-47)	100%	325	12	0%	0%	0%	416
Gagner *et al*[85]	10	3 AMP 4 PDAC	7 (3-14)	100%	NR	22	17%	0%	50%	510

[1]Median; [2]Robotic hybrid; [3]Clavien Ⅲ/Ⅳ. AC: Ampullary cancer; PDAC: Pancreatic ductal adenocarcinoma; AMP: Ampullary adenocarcinoma/ampullary dysplastic adenoma; NR: Not reported; PC: Pancreatic cancer.

9 nodes harvested with the open approach ($P = 0.04$). Again, in this cohort study adenocarcinoma was more commonly found on final pathology in the open group (21% *vs* 4%). Perhaps this difference partially explains the observed results. Unfortunately, margin status was not reported.

LAPAROSCOPIC PANCREATICODUODENECTOMY

Approaching PD laparoscopically is more complex owing to the intricacy of dissection and reconstruction as well as the necessity of multiple critical anastomoses. None-the-less feasibility has been demonstrated[66-70]. The use of a mini-laparotomy and hand-port for creation of the anastomoses is helpful[67,71-75]. MIS approaches are promising, with lower rates of delayed gastric emptying and wound infection when compared to historic open PD controls[16]. Asbun and Stauffer[76] unmatched comparative trial of patients undergoing laparoscopic pancreaticoduodenectomy (LPD) (*n* = 53) and OPD (*n* = 215) demonstrated advantages for LPD in terms of blood loss (103 mL *vs* 195 mL, $P < 0.001$), transfusion requirement (4.7 units *vs* 0.6 units, $P < 0.001$), length of

intensive care unit stay (3 d *vs* 1 d, $P < 0.001$), and total hospital stay (12 d *vs* 8 d, $P < 0.001$). Again, long-term oncologic outcomes have yet to be reported[76]; however R0 resection and lymph node harvest seem sufficient (Table 3)[15,67,69,72,75-85]. It is clear that higher levels of evidence including controlled trials are needed to elucidate clear conclusions.

Resource allocation

In the absence of definitive clinical improvements, feasibility with no poorer results may not justify LPD in light of prolonged operative times, with initial case reports taking upwards of 750 min to completion[10,86]. The learning curve can be overcome in high volume centers, with average operative times decreased to less than 400 min, similar to open PDs (Table 3). Kendrick and Cusati[83], in one of the largest single series available, describe their initial duration of LPD to be 7.7 h, which improved to 5.3 h after approximately 50 cases. Time is money. Mesleh *et al*[87] in an American cost-analysis study comparing open and LPD at a single institution proclaimed that LDP was associated with equivalent overall costs compared with open PD. In their study, operating time and supply costs were higher for LPD; however post-operative admission was more

cost-effective. Neither operative time nor cost should be detrimental to further application of novel MIS approaches in pancreatic surgery.

Oncologic outcomes

PD is mainly utilized for malignant rather than benign disease therefore oncologic safety must be demonstrated prior to widespread application. Gumbs *et al*[16] published a large review that incorporated 27 published papers for a total of 285 cases, of which 32% were adenocarcinoma. Cumulatively, the mean number of lymph nodes harvested ranged from 7 to 36 with a weighted average of 15 nodes. Of the reported margins (174) only 0.4% were positive. Perioperative morbidity and mortality rates were similar compared to open PDs. The study of Kendrick and Cusati[83], described earlier, which included 65 patients who underwent total LPD, 48% for pancreatic adenocarcinoma and 12% for ampullary adenocarcinoma, published outcomes comparable to the open approach. Their study demonstrated an 89% R0 resection with an average of 15 lymph nodes harvested (range 6-31). Recently, 2-year survival rates of 43% and 36% were reported for LDP and OPD respectively[88]. Dulucq *et al*[75] performed a prospective case series of 25 patients, 44% with pancreatic adenocarcinoma and 12% with ampullary adenocarcinoma. They demonstrated a 100% R0 resection with an average lymph node retrieval of 18 ± 5. Gumbs and Gayete[89] found similar results in their experience with the posterior approach, laparoscopic duodenopancreatectomy, retrieving an average of 16 lymph nodes. Unfortunately, resection margins were not reported. Results are encouraging.

A WORD ON ROBOTICS

Robotic-assisted procedures are of interest in pancreatic resection as theoretically they add increased maneuverability, provide precise tissue manipulation, and improve visualization in three dimensions. Disadvantages include loss of tactile feedback, equipment setup and maintenance issues, increased operative times, and associated learning curve. Several studies have presented promising results for robotically assisted DP, with operative morbidity and mortality comparable to other techniques[90-95]. Similarly, supportive evidence exists for combined laparoscopic-robotic and purely robotic PD procedures[95-102]. Additionally, cost does not appear to be as much of a factor as was initially perceived. An American study by Waters *et al*[91] confirmed cost effectiveness for robotic DP. The total cost of robotic DP was $10588, compared to $16059 and $12986 for ODP and LDP respectively. Boggi *et al*[97] reported costs of robotic PD to be an additional 6193 Euros compared to OPD. Yet in its infancy, enthusiasm should not be dampened for robotic pancreatic surgery, as preliminary results are praiseworthy.

CONCLUSION

Current evidence suggests that laparoscopic pancreatic

surgery is feasible and provides benefits over open surgery including decreased blood loss, shorter length of stay, reduced post-operative pain, and expedited time to functional recovery. However, the implementation of MIS approaches to pancreatic adenocarcinoma is limited compared to open approaches. The technical complexity and lack of resectable cases necessary to overcome steep learning curves partly explains the limited utilization by surgeons. Concerns regarding oncologic outcomes may also be implicated. Data on long-term outcomes of tumor recurrence and patient survival are not well defined and ultimately, the success of oncologic operations depends on cancer related long-term survival. Currently laparoscopic pancreatic surgery remains a reasonable surgical option for benign disease and low-grade malignant tumors when performed by expert surgeons in high volume specialty centers. In the future, perhaps after oncologic safety has been well demonstrated, MIS techniques can be recommended for pancreatic adenocarcinoma, as early results are promising.

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2014 ADVANCES IN PANCREATIC CANCER

Novel strategies for managing pancreatic cancer

Welley S Loc, Jill P Smith, Gail Matters, Mark Kester, James H Adair

Welley S Loc, Department of Chemistry, The Pennsylvania State University, University Park, PA 16802, United States

Jill P Smith, National Institute of Diabetes and Digestive and Kidney Disease, The National Institutes of Health, Bethesda, MD 20892, United States

Jill P Smith, Department of Medicine, The Pennsylvania State University, College of Medicine, Hershey, PA 17033, United States

Gail Matters, Department of Biochemistry and Molecular Biology, The Pennsylvania State University, College of Medicine, Hershey, PA 17033, United States

Mark Kester, James H Adair, Department of Pharmacology, The Pennsylvania State University, College of Medicine, Hershey, PA 17033, United States

James H Adair, Departments of Materials Science and Engineering, and Bioengineering, The Pennsylvania State University, University Park, PA 16802, United States

Author contributions: Loc WS contributed by acquisition of data, drafting and writing of manuscript and redrawing figures, performing edits and final approval of submitted manuscript; Smith JP, Matters G, Kester M, and Adair JH contributed to the conception and design, data acquisition, critical and edits, and approval of final version for publication.

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Correspondence to: James H Adair, PhD, Departments of Materials Science and Engineering, and Bioengineering, The Pennsylvania State University, University Park, 223 Hallowell Building, PA 16802, United States. jha3@ems.psu.edu

Telephone: +1-814-8636047 Fax: +1-814-8652917

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Abstract

With the incidence reports of pancreatic cancer increasing every year, research over the last several decades has been focused on the means to achieve early diagnosis in patients that are at a high risk of developing the malignancy. This review covers current strategies for managing pancreatic cancer and further discusses efforts in understanding the role of early onset symptoms leading to tumor progression. Recent investigations in this discussion include type 3c diabetes, selected biomarkers and pathways related to pancreatic intraepithelial neoplasia lesions, drug resistance, and advances in nanomedicine which may provide significant solutions for improving early detection and treatments in future medicine.

Core tip: Pancreatic cancer is currently one of the most aggressive cancers without standard treatment for improving chances of long-term survival. This paper highlights significant research in translational nanomedicine and the challenges in treating pancreatic cancer.

INTRODUCTION

Pancreatic cancer is responsible for over 40000 deaths every year in the United States, representing about 3% of the newly diagnosed cancer cases. However, pancreatic cancer is the fourth most common cause of cancer related death in the United States, predominantly affecting patients ages 60-80 years[1-4]. Pancreatic ductal adenocarcinoma (PDAC) constitutes up to 95% of pancreatic malignancies[4]. Due to poor prognosis and delayed treatment, survival rate during the first year of diagnosis is as low as 20% and decreases to 6% by the fifth year[2]. Reasons for this poor prognosis are related in part to the chemoresistance of PDAC and inability of chemothera-

peutic agents to penetrate the dense fibrotic microenvironment associated with this malignancy. Early detection may improve the outcome and is occasionally possible when small tumors in the head of the pancreas cause obstructive jaundice. However, only 10%-15% of patients are diagnosed in the early stages when surgical resection can be offered[5,6]. Over 90% of subjects are diagnosed with PDAC in the advanced stages[4].

In the past decade, many efforts have been made in translational cancer research, particularly nanomedical avenues, to create novel approaches to drug delivery and understand the early developmental stages of pancreatic cancer. Such advances suggest that stem cell signaling pathways can be used as targets for drug delivery. To date methods of prevention, standard diagnosis, and treatment for pancreatic cancer remain ineffective in improving the survival rate of diagnosed patients. This review covers recent investigations on type 3c diabetes, selected genetic markers, and advances in nanomedicine for early diagnosis of pancreatic cancer.

HISTOPATHOLOGY OF PANCREATIC CANCER

Tumors are classified as invasive ductal carcinoma, intraductal papillary mucinous neoplasm, neuroendrocrine tumors, or islet cell tumors[7]. Invasive ductal carcinoma is referred to as PDAC. Pancreatic cells undergo (1) endoderm formation; (2) pancreatic morphogenesis; and (3) beta cell differentiation to endocrine and exocrine cells[8]. Pancreatic intraepithelial neoplasia (PanIN) lesions are believed to be one of the precursors of PDAC that coincides with multiple successions of genetic mutations[9]. These mutations are possibly provoked by inflammatory stimulus from alcohol abuse or metabolic syndrome[10]. Based on the grade of dysplasia, PanIN lesions can be categorized as type 1A, 1B, 2, or 3, from minimum to severe expansion of immature cells at the ductal epithelium (Figure 1). Genetic defects that follow PanIN-2 and PanIN-3, involve the dysfunction of one or more tumor suppressor genes that result in aberrant signaling pathways driving pancreatic cancer[1,9,11,12].

SYMPTOMS ASSOCIATED WITH PDAC

PDAC can be asymptomatic in the early stages for months or years. Unfortunately, symptoms of pancreatic cancer typically do not manifest until the disease is in an advanced stage[13]. Patients experience a range of symptoms that are not pathognomonic features to pancreatic cancer. Traditional diagnoses highlight notable symptoms, including obstructive jaundice, abdominal and back pain, weight loss, anorexia, dyspepsia, gallbladder enlargement, migratory thrombosis (Trousseaux syndrome), subcutaneous fat necrosis (panniculitis), and hyperglycemia[14,15].

Carcinoma of the head of the pancreas is often detected when small tumors compress the bile duct, resulting in obstructive jaundice in about 75% of subjects[14].

Nausea, vomiting, lethargy and weight loss may also result from change of appetite, bowel habits, and cancer cachexia. While PDAC can cause abdominal and back pain, it is not uncommon for pancreatic cancer patients to have "painless" jaundice where symptoms are not immediately intrusive[15]. On occasions, tumors of the pancreas invade the superior mesenteric vessels or splenic vein resulting in hemorrhage from varices.

Collective evidence supports the claim that type 3c diabetes is pancreatogenic diabetes and can be caused by chronic pancreatitis due to loss of functioning pancreatic islet cells or may occur as the result of a paraneoplastic phenomenon caused by pancreatic cancer. While further studies are needed to distinguish cancer-induced diabetes from diabetes caused by other exocrine pancreatic diseases, they are classified as two different types of diabetes mellitus by the American Diabetes Association[16,17]. This paraneoplastic syndrome precedes most cancer-specific symptoms by several months or years before tumors become radiologically detectable[17]. New-onset diabetes may also increase the likelihood of pancreatic cancer by 5 to 8 times, with approximately 1% of patients developing the cancer within three years. Progressive and unintentional weight reduction is associated with type 3c diabetes[18]. Unlike type 2 diabetes that is associated with weight gain and obesity, patients with type 3c continue to lose weight as glycemic control worsens in parallel with cancer advancement (Figure 2A). Weight loss is an early event of type 3c that is attributed to either cachexia or loss of adipose tissue[17]. Cachexia is a chronic physical wasting and malnutrition disease that results in more than 10% body weight loss in late cancer stages. When cachexia is absent or has yet to occur, patients rapidly lose weight by adipose tissue inflammation from interactions with pancreatic cancer (Figure 2B). Inflammation in adipose tissue can contribute to peripheral insulin resistance by altering adipocyte secretion and propagate pathogenic processes similar to type 2 diabetes. About 90% of the hormonal secretion from adipose tissue macrophages is comprised of inflammatory cytokines[17,19]. The accumulation of inflammatory cytokines triggers abnormal adipocyte secretion and reduced hepatic insulin sensitivity. This reaction leads to an increase of leptin levels (related to the loss of appetite) and decrease in adiponectin. Leptin and adiponectin are primary precursors to insulin resistance in type 2 diabetes[20-22], which can be regulated by limiting glucose intake and weight gain. Diet changes, such as reduction in carbohydrate intake, have minimal effect on pancreatic cancer-induced diabetes. Weight loss symptoms and diabetes will persist until the tumors are resected. One potential mediator of the cancer-associated diabetes is the over-expression of a pluripotent hormone adrenomedullin that mediates insulin resistance through the interaction of adrenomedullin receptors on β-cells[17]. An increase in endogenous expression of adrenomedullin results in β-cell dysfunction which inhibits insulin secretion in the plasma and tumors.
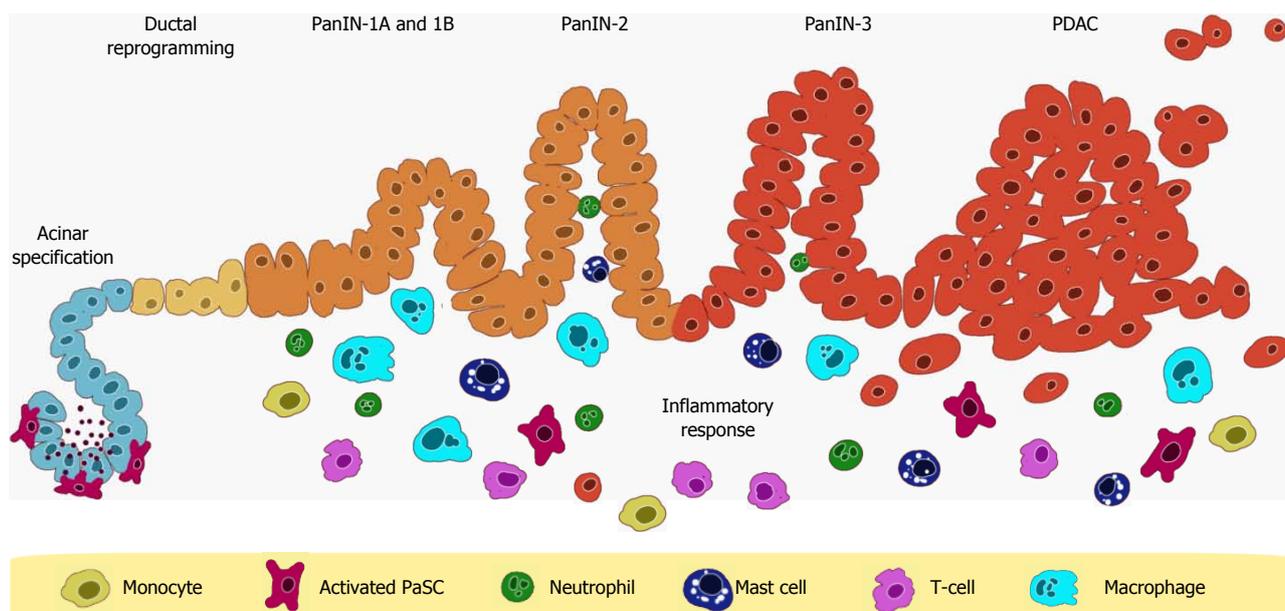
Figure 1 Example of pancreatic intraepithelial neoplasia lesion development to pancreatic ductal adenocarcinoma. Inflammatory stimuli trigger the activation of pancreatic stellate cells (PaSC) surrounding acinus cells. Inflammatory cells (monocytes, T-cells, neutrophils, mast cells, and macrophages) gather in response and release ligands (interleukin-6) that activate *STAT3* gene to promote pancreatic intraepithelial neoplasia (PanIN) development in susceptible tissue with oncogenic mutations such as the Kras. PDAC: Pancreatic ductal adenocarcinoma. Figure redrawn with permissions from Elsevier: [10] and Macmillan Publishers Ltd: [12].

The relationship between diabetes and pancreatic cancer has been studied since the early 1830s, but the biological significance of type 3c diabetes in relation to pancreatic cancer had not been acknowledged until recently[23]. Early diagnosis of type 3c could potentially lead to early diagnosis and treatment of patients with pancreatic cancer months to years before the tumor appears radiologically. Early distinction between type 2 and type 3c diabetes requires a high level of awareness and expertise, and may lead to the earlier diagnosis of pancreatic cancer. Severe weight loss is also intimately associated with a variety of cancers and occasionally occurs several months prior to death[24]. Thus, understanding the collective effects of type 3c is substantial in distinguishing pancreatic cancer from the diverse array of malignancies.

DIAGNOSTIC TESTS AND BIOMARKERS

Currently there are no adequate diagnostic tests for early detection of pancreatic cancer, and routine radiographic tests or endoscopic ultrasound screening is only recommended for those individuals with a family history of pancreatic cancer, chronic pancreatitis, precancerous lesions, or new-onset diabetes. Serological markers such carbohydrate antigen (CA)19-9, MIC-1, carcinoembryonic antigen, human chorionic gonadotropin β, and CA72-4 have also been of interest but lack sufficient sensitivity and specificity for effective early cancer detection[25-28].

However, research continues to make progress on uncovering genetic markers that are responsible for notable pancreatic cancer cell phenotypes. Recently, the role of mucin-1 (MUC-1) in malignant cells was first reported to upregulate multi-drug resistance genes such

as *ABCC1*, *ABCC3*, *ABCC5* and *ABCB1*[29]. MUC-1 is a transmembrane glycoprotein that lines the apical surface of epithelial cells, normally present to protect the body from infectious pathogens. Overexpression of MUC-1 is found in patients with common cancers that include pancreatic[30], breast[31], ovarian[32] and thyroid[33] cancers. MUC1 overexpression may be and enabled by the phosphatidylinositol 3'-kinase/Akt signaling pathway, a pathway associated with chemotherapeutic drug resistance in other cancers[34].

GENETIC MUTATIONS ASSOCIATED WITH PANCREATIC CANCER

While there are at least 25 altered genes related to cancer pathways (*i.e.,* cell adhesion, apoptosis, and replication), only a handful have been identified in pancreatic cancer studies[35,36]. BCRA2 mutations are found in up to 10% of those with PDAC. A germline variant of the cholecystokinin-B gene has been identified in over 35% of patients with PDAC and predicts both risk and survival[28]. Activated Kirsten-Ras (Kras) oncogene is harbored in > 95% of pancreatic cancer tumors and is critical in cell proliferation and apoptotic resistance to hostile microenvironments (in the presence of anti-cancer agents)[1]. Activation of the Kras oncogene releases Ras proteins that initiate mitogen-activated protein (MAPK) cascades[37-39]. MAPK participates in many critical cellular events, including cell division, response to surroundings, movement, and cell death. Mutated Kras is accepted as a "Driver" gene for pancreatic cancer that propagates a series of ongoing cellular signal transduction processes that cause uncontrollable proliferation and architectural
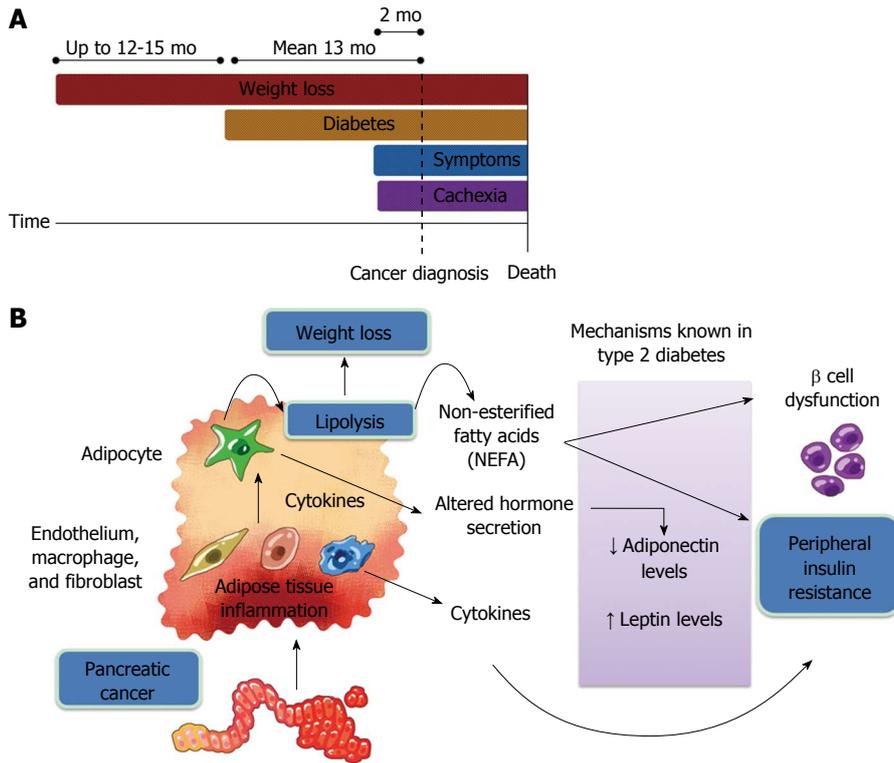
Figure 2 Symptoms of paraneoplastic type 3c diabetes preceding pancreatic cancer. A: A comparison of weight-loss timeline to cancer-specific symptoms; B: Schematic representation of the cause for progressive weight reduction and insulin resistance. Adipose tissue inflammation triggers an alteration of adipocyte secretion and propagates pathogenic processes similar to type 2 diabetes, eventually leading to cachexia. Figures redrawn with permission from Macmillan Publishers Ltd: [17].

abnormalities where acinar tissue is replaced with ductal lesions.

Kras mutations are also capable of reducing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) sensitivity[40]. Abnormalities are likely to occur at codon 12 (G12D), involving a point mutation of one glycine to aspartic acid (G12D), or glycine to valine (G12V). TRAIL is a transmembrane protein that can be proteolytically cleaved from the cell surface to mediate apoptosis and anti-tumor activities[40]. Inhibitors that directly target oncogenic Kras have not yet been developed, but remain an active area of investigation. However, Kras mutations can trigger an enrichment of a cytokine receptor, osteoprotegerin (OPG), which directly inhibits TRAIL solubility and potentially induces apoptosis[40,41]. Interestingly, increased OPG and TRAIL levels are also found in subjects with type 2 diabetes mellitus[42], but connections to type 3c diabetes have not been implicated.

SIGNALING PATHWAYS ACTIVATED IN PANCREATIC CANCER

Oncogenes depend on various signaling pathways to initiate tumor formation. Since most attempts to directly inhibit oncogenes like Kras have failed, attention has shifted to other critical signaling pathways for targeted cancer therapy[43]. The Notch pathway, for instance, exerts its biological influence by maintaining homeostasis

during embryonic development in multicellular organisms[44] and is important in development of the pancreas. The loss of Notch signaling in the pancreas results in premature differentiation of endocrine and exocrine cells. Therefore, this pathway is essential for determining the fate of functioning pancreatic cells in epithelial and non-epithelial tissues. However, controversy exists in literature as to whether the Notch pathway serves as a promoter for tumor progression or an inhibitor[45,46]. Lateral inhibition mechanisms of the Notch pathway involve a group of receptors (Notch1, Notch2, Notch3, and Notch4), target, and ligand key components that contribute uniquely to PanIN progression[46]. For example, deletion of the Notch1 receptor generally accelerates PanIN lesion development and lowers median survival in Pdx1-Cre^{ERT2}; LSL-Kras^{G12D}, Pdx1-Cre;Kras^{G12D}, and Ptf1a-Cre; Kras^{G12D} mouse models[46-49]. The loss of the Notch2 receptor in Ptf1a-mouse models, however, halts lesion progression and increases chances of survival[49]. Tumor inhibition was also reported in several studies where the up-regulation of Hes1 from activated Notch pathway suppresses the expression of p57, which prevents progenitors from undergoing premature differentiation and uncontrollable proliferation[50]. Without harming healthy adult cells, tumor suppression was achieved in zebrafish by forcing exocrine pancreatic precursors through Notch signaling to inhibit acinar cell differentiation[45]. Ongoing investigations on type 2 diabetes also imply that the Notch pathway is responsible for insulin-resistance in pancreatic cells

Table 1 Desired characteristics for a nanoparticle drug-delivery platform

Desired characteristic	Comments
Inherently non-toxic materials and degradation products	The initial material selection should be based on non-toxic materials especially with an aim toward human health care
Small size (10–200 nm)	There is not a particular size that seems most efficacious, particularly based on *in vivo* studies. This is the range of particle diameters that have proven most effective for a wide variety of delivery systems. Also of note is the debate around the influence of particle shape[83]
Encapsulation of active agent	To be effective, the active agent must be encapsulated within the nanoparticle vehicle. Surface decoration (*i.e.*, adsorption) will often be effective *in vitro* but falls short for *in vivo* studies because of the reticulum endoplasmic systems *in vivo*
Colloidally stable in physiological conditions	The nanoparticle vehicle and surface functionalization must resist agglomeration for the solution pH values, ionic strength, macromolecular interactions, and temperature encountered in the physiological environment
Clearance mechanism	The nanoparticle vehicle must have a ready clearance mechanism to avoid the cumulative and/or systemic effects of the drug-laden particles
Long clearance times	Resistance to agglomeration and other effects that remove the nanoparticle-encapsulated drug from the patient must be avoided to promote long circulation times in the circulatory system for as many of the nanoparticles to find and sequester in the cancer cells as possible
Biologically or extrinsically controlled release of therapeutic agents	There should be a trigger mechanism such as the acidic pH within the tumor or during endosome maturation designed into the nanoparticle platform to ensure the release of the encapsulated drug into the targeted tissue
Can be targeted to cell/tissue of choice	The nanoparticle platform should be capable of surface bioconjugation to target molecules for the specific cancer to provide the greatest uptake with the lesions and fewest least side effects with healthy tissue

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(from the expression or inactivation of *Hes1* gene, Rbp-Jk protein ligand, and *Ngn3* gene)[51]. The function of the Notch pathway during PDAC development is dependent on the targeted receptor and the genes expressed. Clearly, Notch signaling pathway targeted therapy serves as a potential target for treating pancreatic cancer. Such therapies must be exercised with caution since a wide variety of cells rely on ligand-dependent pathways for growth and survival.

Another important pathway activated by GTP-protein coupled receptors (GPCRs) is the PI3 kinase signaling pathway that phosphorylates Akt and activates downstream mTOR and subsequent proliferation. In a large GWAS study, GPCRs were found to be the most frequent signaling pathways involved in PDAC[52]. One GPCR, the cholecystokinin (CCK) receptor is over-expressed and ubiquitous in PDAC[53]. Stimulation of the CCK receptor accelerates PanIN development in the Kras transgenic mouse model[54]. Targeting the CCK receptor has become important in new therapeutics for PDAC and indeed if this receptor is down-regulated growth is inhibited and downstream signaling through PI3 kinase is blocked[55].

TREATMENT

Treatment options for PDAC patients that present in the late stages are limited to chemotherapy and radiation. Conventional chemotherapeutic agents are ineffective against PDAC for several reasons among which include the microenvironment. PDAC tumors are highly fibrous and poorly vascularized[13] prohibiting adequate penetration of the tumor by chemotherapeutic agents. The heterogeneous nature of cancer cells and tissue hypoxia is associated with drug resistance, often requiring higher drug dosages during treatment and increased toxicity such as peripheral neuropathy, bone marrow

toxicity, and cardiotoxicity. Gemcitabine is the gold standard for advanced PDAC, but only affords survival up to six months[56,57]. Survival with gemicitabine is, however, improved when administered with other agents[58]. Capecitabine and 5-fluorouracil[59,60] are also common antimetabolites administered in clinical trials as a standard single-drug treatment[61]. These agents have been used in conjunction with platinum-based agents and other cancer drugs such as leucovorin, exactecan, and irinotecan[5]. Radiation therapy is recommended in conjunction as an adjuvant and a chemosensitizer[62,63]. Clinical trials that administer combined drug therapy such as FOLFIRINOX (5-fluorouracil with leucovorin, irinotecan, and oxaliplatin) have shown greater efficacy for metastatic cancer, but with profound limitations due to systemic toxicity and neurotoxicity[64-67]. Recently, survival of PDAC patients has been marginally improved by using a combination of nab-Paclitaxel plus gemcitabine[58].

Patients may be offered surgery in absence of metastatic spread as determined by positron emission tomography, magnetic resonance imaging, and triphasic computed tomography scans. The Whipple operation is performed on pancreatic cancer involving the head of the pancreas if the superior mesenteric vessels are not affected[6,68]. Pylorus preserving Whipple operation involves removing the first section of the duodenum while others may undergo the standard Whipple operations which involve the removal of a part of the stomach. Adjuvant chemotherapy and radiation therapy usually follow the resection in an attempt to decrease relapse rates. Preoperative chemotherapy and radiation therapy can sometimes restage tumors and make them amenable to surgical resection, but patients with locally advanced pancreatic cancer from the body and tail of the pancreas are often not qualified for surgery due to metastatic spread to the celiac artery. Evidently, surgery assures the longest survival, but

Table 2 The selection criteria for nanomaterial drug delivery systems

Nano particulate material	Size (nm)	Therapeutic agent(s) carried	Advantages	Limitations
Biodegradable polymers	10-100	Plasmid DNA, proteins, peptides, low molecular-weight (MW) organic compounds	Sustained localized drug delivery for weeks	Exocytosis of undissolved nanoparticles. Fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities
Ceramic	< 100	Proteins, DNA, chemotherapeutic agents, high MW organic compounds	Easily prepared, water dispersible, stable in biological environments	Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation
Metals	< 50	Proteins, DNA, chemotherapeutic agents	Small particles present a large surface area for surface decoration delivery	Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation
Polymeric micelles	< 100	Proteins, DNA, chemotherapeutic agents	Suitable for water-insoluble drugs due to hydrophobic core	Toxicity of materials, fixed functionality after synthesis
Dendrimers	< 10	Chemotherapeutic agents, anti-bacterial, anti-viral agents, DNA, high MW organic compounds	Suitable for hydrophobic or hydrophilic drugs	May use toxic materials, time consuming synthesis, fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities
Liposomes	50-100	Chemotherapeutic agents, proteins, DNA	Reduced systemic toxicity, increased circulation time	Fixed functionality after synthesis, some leakage of encapsulated agent, lack of colloidal stability
3D printing	20-2000	Chemotherapeutic agents, proteins, DNA, imaging agents	Precise control over size, shape, and surface functionalization. 3D printing can be used with an array of processing techniques to create porous scaffolds[85] and lab-on-chip devices[86] for personalized medicine[87]	Toxicity of materials depending on material
Calcium phosphosilicate	20-60	Chemotherapeutic agents, RNA, high and low MW organic compounds, imaging agents	Simple preparation, suitable for hydrophilic or hydrophobic drugs, colloidal stability in physiological environments, pH-dependent dissolution results in intracellular delivery of drugs, composed of bio-resorbable material	Encapsulated materials limited to solubility in water or organic solvent

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promise for full recovery from advanced PDAC is not yet feasible[69,70].

FUTURE DIRECTIONS BASED ON NANOMEDICINE

Novel approaches for pancreatic cancer therapy are desperately needed. The trinity offered by nanomedical approaches to simultaneously seek, treat, and track human cancer is slowly emerging from the basic nanoscience toward clinical deployment to treat pancreatic cancer. Adair *et al*[71] reviewed the selection criteria for drug delivery strategies based on several nanomaterial platforms. The selection criteria for nanomaterial drug delivery systems are summarized in Tables 1 and 2. Novel strategies using nanotechnology research may lead to advantages in early detection via bioimaging, specific targeting of cancer cell receptors and effective treatment with lower side effects and drug degradation. There is a great demand to improve current drug delivery procedures to overcome drug resistance without causing serious off target toxicity. Targeting proteins involved with signal transduction is one strategy that is currently under investigation. Studies in growth factor inhibitors (opioid growth factors)[72,73] for biotherapy and biocompatible nanomaterials for drug carrier systems, introduce promising directions towards effective cancer management despite limitations. Novel biomarkers like Plectin-1 (Plec1)[74] have been found to be useful in the early detection of small pre-invasive PanIN Ⅲ lesions and metastases. Such biomarkers provide an advantage in early detection when they are over-expressed in specific organs. It was shown that Plec1 can also be used to safely distinguish PDAC from benign conditions and thus this method is more effective than cross-sectional abdominal and invasive endoscopic imaging techniques. Although a cure for PDAC is not feasible with currently available treatment, research in the next decade will develop better prevention and prognosis modalities to diagnose PDAC and improve chances of survival.

One of the most promising nanomedical approaches reported in recent years is based on a novel material system, calcium phosphosilicate hydrate nanoparticles (CPSNPs), in which encapsulated imaging agents and/or drugs, can be delivered in a targeted manner to a variety of cancers including pancreatic cancer[71,75-80]. For example, Barth *et al*[81] have demonstrated that a FDA-approved near infra-red fluorophore, indocyanine green (ICG), also known as Cardio-Green™, when encapsulated in the CPSNPs, can be used as a theranostic (*i.e.*, a combined diagnostic and therapeutic) agent for a variety of cancers based on a new cancer diagnosis and treatment strategy designated as photo-immuno nanotherapy (PINT). PINT results resurrection of the immune response of the host animal, permitting the immune system to fight the cancer

directly. In an earlier report, Barth *et al*[82] also demonstrated that gastrin-10 can be used for targeted delivery of ICG-encapsulated CPSNPs *in vivo* based on an orthotopic graft of a human pancreatic cancer in the athymic murine model. The trigger to release the chemotherapeutic agent is inherent dissolution of the CPSNPs in either the acidic local pH in the fluid surrounding many solid tumor types or, after endosomal uptake of the drug-laden CPSNPs into cancer cells, the decreased pH associated with maturation of endosome to endo-lysosomes. Targeting permits the PINT to be used for efficacious uptake in solid tumors and, in an unprecedented fashion, for non-solid tumor cells such as chronic myeloid leukemia[78,81,82]. Thus, the combination of early detection with more efficacious delivery and more effective treatment promised by nanomedical approaches is emerging as a viable alternative for pancreatic cancer diagnosis and treatment.

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2014 ADVANCES IN PANCREATIC CANCER

Robotic surgery of the pancreas

Daniel Joyce, Gareth Morris-Stiff, Gavin A Falk, Kevin El-Hayek, Sricharan Chalikonda, R Matthew Walsh

Daniel Joyce, Gareth Morris-Stiff, Gavin A Falk, Kevin El-Hayek, Sricharan Chalikonda, R Matthew Walsh, Department of General Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States
Author contributions: Joyce D and Morris-Stiff G contributed to the study idea, study design, literature search and preparation of the manuscript; Falk GA, El-Hayek K, Chalikonda S and Walsh RM contributed to the study design and revision of the manuscript.
Correspondence to: Daniel Joyce, Resident Physician, Department of General Surgery, Digestive Disease Institute, Cleveland Clinic, Cleveland, OH 44195, United States. joyced4@ccf.org
Telephone: +1-216-4457576 Fax: +1-216-4457653
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Abstract

Pancreatic surgery is one of the most challenging and complex fields in general surgery. While minimally invasive surgery has become the standard of care for many intra-abdominal pathologies the overwhelming majority of pancreatic surgery is performed in an open fashion. This is attributed to the retroperitoneal location of the pancreas, its intimate relationship to major vasculature and the complexity of reconstruction in the case of pancreatoduodenectomy. Herein, we describe the application of robotic technology to minimally invasive pancreatic surgery. The unique capabilities of the robotic platform have made the minimally invasive approach feasible and safe with equivalent if not better outcomes (*e.g.*, decreased length of stay, less surgical site infections) to conventional open surgery. However, it is unclear whether the robotic approach is truly superior to traditional laparoscopy; this is a key point given the substantial costs associated with procuring and maintaining robotic capabilities.

Core tip: This invited article reviews the latest developments in robotic surgery of the pancreas in a clear and succinct manner. It highlights the merits of robotic surgery while explaining the challenges that physicians face when integrating new technology into clinical practice.

INTRODUCTION

Pancreatic surgery remains one of the most challenging and complex fields in general surgery. Kausch[1] performed the first pancreatoduodenectomy (PD) in 1909, with the operation later being popularized by Dr. Allen Oldfather Whipple, though he only performed 37 such operations in his career. Nevertheless, the operation did not gain widespread acceptance until the 1980s having gained notoriety as a dangerous and morbid operation, which was associated with a 30% perioperative mortality rate[2]. The centralization of pancreatic surgery to high volume referral centers has led to a reduction in the perioperative mortality rate to less than 5% for PD[2-5]. Despite improvements in technique and perioperative care, major pancreatic resections carry an appreciable rate of major morbidity with high volume centers reporting a 30%-40% morbidity rate for PD[3-5]

Minimally invasive surgical approaches are becoming the standard of care for many abdominal operations given their superiority over open procedures in terms of surgical site infection, postoperative pain, and length of stay while providing equivalent oncologic outcomes in

cases of malignancy[6]. The laparoscopic approach is now considered the standard of care for cholecystectomy, anti-reflux surgery, colon cancer, and bariatric surgery. It is worthwhile considering the concept of surgical oncotaxis in patients with pancreatic cancer, one of the most aggressive solid organ malignancies. This concept proposes that surgical stress can depress the anti-tumor immune response and foster tumor progression[7]. Kondo *et al*[8] demonstrated a reduction in the incidence of systemic inflammatory response in patients undergoing minimally invasive PD. In addition, earlier and improved recover may lead to more patients receiving adjuvant therapies or being enrolled into clinical trials.

It is now almost twenty years since Gagner and Pomp[9] described the first laparoscopic PD, however, it has not gained widespread popularity. This has been attributed to the retroperitoneal location of the pancreas, its close relationship with major vascular structures, and the tedious nature of the dissection required to optimize oncological margins in pancreatic cancer. Perhaps the largest barrier of all to laparoscopic PD is the challenge of reconstruction since three separate anastomoses are required. This is illustrated by the more widespread acceptance of distal pancreatectomy (DP) in recent years since there is no reconstruction required[10]. However, the laparoscopic approach to DP may itself be under-utilized[11]. The slow adoption of minimally invasive pancreatic surgery, particularly PD, mirrors that of prostatectomy. Laparoscopic prostatectomy never gained popularity due to its technical complexity and steep learning curve, reported to be in the range of 150 cases, when assessed in terms of blood loss and operating time. In fact, it may require a staggering 700 cases to reach expert proficiency in maximizing potency outcomes in patients undergoing prostatectomy[12]. In 2001, prostate surgery was revolutionized by the introduction of the robotic surgery. In less than eight years, robotic assisted prostatectomy has become the most common approach to prostatectomy in the United States with over 60% of prostatectomies being performed with robotic assistance[13].

The development of robotic surgery was spurred by an interest in the military to perform operations remotely such as near the battlefield, or in space[14]. While much of the early work was completed by the US Department of Defense, the current and only robotic surgery platform has been brought to the marketplace by Intuitive Surgical®. The DaVinci® surgical system consists of a three or four-armed robot which is operated by the surgeon who sits at a separate console. The robotic platform overcomes many of the key shortcomings of traditional laparoscopy that include monocular vision, limited degrees of freedom and the effects of pivot and fulcrum, which make suturing in particular difficult to master. In contrast, the robotic approach affords the surgeon a three-dimensional stereoscopic view of the operating field and restores hand-eye coordination that is often lost in traditional laparoscopy when the camera is offset to the plane of dissection. Endowrist®

(Sunnyvale, CA) instrumentation not only replicates the movements of the human hand with seven degrees of freedom but also eliminates hand tremor (Figure 1A). The key triumph of the robotic platform over traditional laparoscopy is the ease with which one can suture and tie intracorporeal knots independent of the operative setup. The robotic approach is not without disadvantages. The lack of haptic feedback has been cited as a possible drawback since its absence may lead surgeons to place excessive tension whilst tying sutures leading to tearing of the tissues being sutured[15]. The robotic platform is expensive with an initial capital cost of $1-2.5 million; annual maintenance liabilities well over $100000, and many of the instruments are single use only[16].

This review discusses the impact of robotic technology on pancreatic minimally invasive surgery.

ROBOTIC PANCREATODUODENECTOMY

Giulianotti *et al*[17] first performed robotic assisted PD in 2001, and the initial series of 8 patients demonstrated that robotic PD was feasible and safe, reporting a 37.5% morbidity rate and one perioperative death (due to Boerhaeve's Syndrome). The mean operative time in this series was 270 min. Surgeons have adopted robotic assistance for minimally invasive PD with much more enthusiasm than the traditional laparoscopic approach. Indeed the authors of the first reported laparoscopic PD series concluded at one point that there was no benefit to the minimally invasive approach to PD[10,18]. In 2012 it was reported that only 7 centers worldwide had an experience of 30 or more patients who had undergone laparoscopic PD[19], somewhat meager progress for an operation initially described eighteen years earlier.

On the contrary, robotic assisted PD while not widespread is being reported with increasing frequency. This mirrors the development of minimally invasive prostatectomy; robotic assistance seems to act as an enabler to surgeons who do not feel comfortable performing the operation with conventional laparoscopic techniques. The reported approaches for robot assisted PD vary, some groups adopt a hybrid laparoscopic/robotic approach while others perform the entirety of the operation robotically. Narula *et al*[20] report a hybrid laparoscopic and robotic approach for PD, the authors complete the dissection laparoscopically and employ the robot to perform the reconstruction taking advantage of the precision and dexterity of the robot for placing sutures (Figure 1B). Fernandes *et al*[21], the initial pioneers of the robotic PD advocates a full robot-assisted approach believing that "there is no role for a hybrid hand-assisted or laparoscopic/robotic approaches".

It is reasonable to conclude that robot assisted PD is a safe operation and can be performed with a rate of morbidity and mortality equivalent to open PD. Several series demonstrate a reduction in blood loss and a trend towards reduced length of stay (LOS) *vs* the open operation, for example the most recent series published
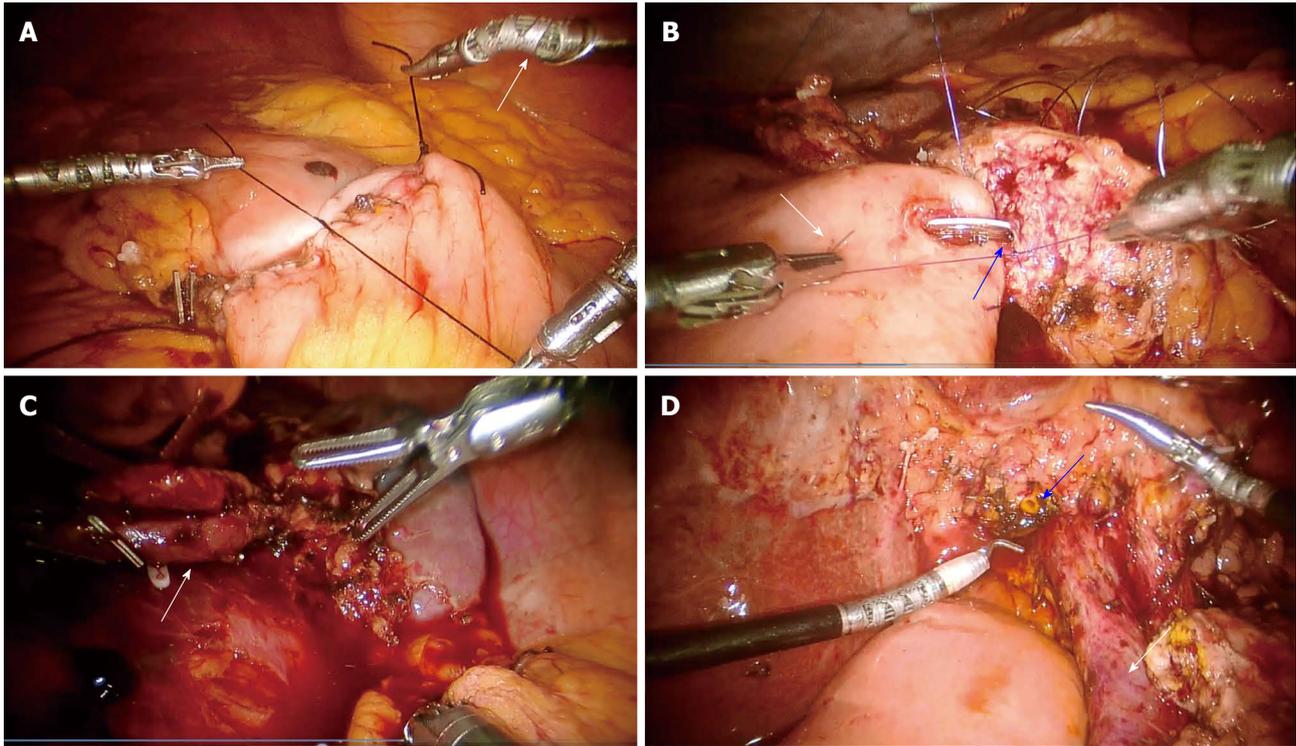
Figure 1 Robotic surgery of the pancreas. A: Completion of the second layer of the anterior duodenojejunal anastomosis following pancreatoduodenectomy. The white arrow highlights the endowrist® capabilities of the robotic arms; B: Completion of the posterior row of the pancreaticojejunal anastomosis with intracorporeal knot tying. The white arrow indicates the jejunal limb with the blue arrow pointing to a pediatric feeding tube entering the main pancreatic duct; C: Splenic hilum following robotic spleen preserving distal pancreatectomy. The excellent visualization and advanced endowrist® technology allow for a precise dissection of the splenic artery and vein (white arrow); D: Hepatic hilum (common hepatic duct indicated by blue arrow, portal vein indicated by white arrow) following resection of pancreatic head and duodenum.

describes the operation in 132 patients with a mean EBL of 527 mL and an average LOS of ten days. Comparative studies detailing open PD *vs* the robotic approach report reduced blood loss with the latter approach[22-24]. PD is an operation associated with morbidity rates of 30%-50%; robotic surgery appears at least equivalent to open surgery in terms of morbidity and mortality. A recent meta-analysis of robotic *vs* open pancreatectomy favored the robotic approach with a risk difference of 12% for morbidity between open and robotic approaches[25]. Of course, one has to be mindful that many of the studies involved may have a selection bias favoring the robotic approach, with surgeons choosing small and more favorable periampullary tumors for cases early in their robotic series. That said, these tumors are often found within the setting of a soft pancreas and a small duct, and thus pancreatic fistulae may be expected more commonly in these patients. Furthermore, the robotic approach may lead to surgery being performed in patients with a high body mass index that previously may have not been considered for an open approach. The morbidity of PD is largely driven by the incidence of pancreatic fistula. Once again the robotic approach compares favorably with contemporary series of open PD, with rates varying from 6%-35% (Table 1). However, the data must be interpreted with caution since the definition of the presence and severity of pancreatic fistulae is not uniform across published reports, moreover some of the initial series did not perform a pancreaticojejunostomy and opted for pancreatic duct closure.

PD is primarily performed for periampullary and pancreatic carcinomas hence the oncologic outcome of the resection is a key measure by which one should judge the success of one approach over the other. All series report acceptable rates of a microscopically negative "R0" resection (80%-100%), and an adequate lymph node harvest. The systematic review by Cirocchi *et al*[26] included thirteen case series with an average R1 resection rate of 9%. Another systematic review reported a greater lymph node harvest with the minimally invasive approach to PD[27]. These impressive results must be interpreted with caution since many surgeons tend to opt for the open approach in patients with larger more extensive tumors while preferring the robotic approach in patients with more oncologically favorable ampullary and duodenal malignancies. It is also important to consider the new staining techniques used to assess resection margins, in particular the retroperitoneal margin, as these now show that R0 rates are less than previously accepted, and hence future series will need to use this methodology so that accurate comparisons of oncological benefits of robotic surgery may be assessed[28].

In summary robot assisted minimally invasive approach to PD appear to lead to a reduction in operative

Table 1 Outcomes of robot assisted pancreatoduodenectomy									
Ref.	*n*	OR time (mean)	EBL (mean)	LOS (mean)	RO	LN harvest (mean)	Conversion	Fistula	Morbidity	Mortality
Giullianotti *et al*[40]	60	421	394	22 (5-85)	Italy: 100% United States: 89%	Italy: 21 United States: 15	18%	31.30%	NR	1.5%[1]
Buchs *et al*[46]	41	431.5	389	12.7	NR	NR	4.80%	19.50% (4A/3B/1C)	39%	2.40%
Narula *et al*[20]	5	420	NR	9.6	NR	16	37.50%	0	NR	0%
Zhou *et al*[24]	8	718	153	16.4	100%	NR	0%	25%	NR	0%
Zeh *et al*[47]	50	568	350	10	89%	18	16%	20% (5A/2B/4C)	26% I / II 30% III /IV	2%
Chalikonda *et al*[22]	30	476	485	9.79	100%	13.2	10%	6.60%	30%	3%
Lai *et al*[23]	20	491.5	247	13.7	73.30%	10	5%	35%	50%	0%
Zureikat *et al*[36]	132	527	NR	10	NR	NR	8%	17% (12A/5B/5C)	41% I / II 22% III /IV	30 d: 2% 90 d: 5%

[1]Included other pancreatic cases. NR: Not recorded; LOS: Length of stay; EBL: Estimated blood loss; LN: Lymph node; OR: Operating.

blood loss and reduced length of hospital stay. In addition, the approach appears equivalent to open surgery in terms of short-term oncologic outcome, and both morbidity as well as mortality. However, these outcomes must be interpreted carefully given the retrospective nature of the data and the real possibility of selection bias in favor of those patients considered for the robotic approach to PD.

ROBOTIC DISTAL PANCREATECTOMY

Laparoscopic distal pancreatectomy (DP) has been adopted with much more enthusiasm than PD; this is unsurprising since there is no reconstruction involved. It can be performed safely and affords the patients several advantages including less blood loss, fewer complications, less pain and a reduced length of stay[22,29] (Table 2). A multicenter comparative study evaluated 142 laparoscopic and 200 open DPs, demonstrating less blood loss for the laparoscopic technique (357 mL *vs* 588 mL), an overall reduction in complications (40% *vs* 57%), and a reduced LOS (5.9 d *vs* 9 d)[30]. Nonetheless the laparoscopic approach may be limited by a lower spleen preservation rate and a higher rate of unplanned splenectomy[31]. Others cite concern for the oncological adequacy of laparoscopic DP given the reduced dexterity associated with the laparoscopic approach. Despite the acceptance of laparoscopic DP in the literature it has not been widely applied; NSQIP data for 2005-2010 reports that 27% of DP cases are performed laparoscopically[11].

The robotic approach to distal pancreatectomy is also gaining popularity. Daouadi *et al*[32] reported on a retrospective series of robotic and laparoscopic DPs (30 *vs* 94 respectively), the results were significant for a reduced conversion rate to open surgery (0% *vs* 16%), a trend toward fewer incomplete resections (non R0 resections), and a shorter operative time (293 min *vs* 371 min). This is in contrast however to the majority of reports which report a longer operative time for robotic DP[26]; this may be explained by the groups extensive experience with the robotic approach.

Splenic preservation is indicated for benign and low-grade pancreatic tumors; the benefits of which are highly significant with a reduction in infectious and overall complications[33] (Figure 1C). Two groups have demonstrated high rates of splenic preservation with the robotic approach; Hwang *et al*[34] successfully preserved the spleen in 21 of 22 cases (95.5%), and the Indiana University group reported splenic preservation rates of 65%, 12% and 29% for robotic DP, laparoscopic DP and open DP respectively[35] in a retrospective series with unspecified selection criteria. This paper also reported a reduced LOS for the robotic group (3.8 d) as compared to 7.7 and 6.4 d for the open and laparoscopic groups respectively, the savings associated with this leading to the robotic approach being comparable from a cost standpoint.

The morbidity of robotic DP is at least equivalent to its open and laparoscopic counterparts. Zureikat *et al*[36] reported a 43% (27A, 10B, 4C) pancreatic fistula rate in a series of 83 patients undergoing robotic DP. Other groups report lower fistula rates with the meta-analysis performed by Cirrochi *et al*[37] reporting a 16.9% fistula rate, however, not all groups reported pancreatic fistulae according to the ISGPF definition. Minimally invasive DP is also associated with a lower surgical site infection rate in comparison to the open approach; this of course is not exclusive to the robotic approach and applies equally to the traditional laparoscopic approach[38].

In summary, robot assisted DP is indeed feasible and safe with distinct advantages over open surgery including a reduced length of stay and overall complication rate. Additionally the rate of splenic preservation appears to be far superior to the open and laparoscopic approach. However, it is questionable if the robotic approach affords the patient additional advantages over traditional laparoscopic DP particularly when splenic preservation is not indicated.

OTHER PROCEDURES

While malignancy remains the most frequent reason for pancreatic surgery, many patients undergo resection or

Table 2 Outcomes of robot assisted distal pancreatectomy

Ref.	*n*	OR time (mean)	EBL	LOS (mean)	Conversion	Fistula	Morbidity	Mortality
Waters *et al*[35]	17	298	279	4	12%	0	18%	0
Kang *et al*[48]	20	298	372	7.18	NR	NR	10%	0
Daoudi *et al*[32]	30	293	212	6.1	0	46% (6A/4B/4C)	46% I / II	0
							20% III /IV	
Giulianotti *et al*[40]	46	331	323	9.3	6.5%	20.9%	NR	NR
Zureikat *et al*[36]	83	256	NR	6	2%	43% (22A/10B/4C)	60%	0

NR: Not recorded; LOS: Length of stay; EBL: Estimated blood loss; LN: Lymph node; OR: Operating.

drainage procedures for chronic pancreatitis. The benefits of a robotic approach may be even more applicable to this cohort of patients who have a benign disease and are primarily undergoing surgery to ameliorate pain and improve their quality of life. The major resectional procedures have been discussed in detail above.

The versatility of the robotic platform is illustrated by a series of case reports documenting less common procedures in patients with chronic pancreatitis. Peng *et al*[39] reported four cases of robot-assisted duodenum preserving pancreatic resection while Zureikat *et al*[36] reported three Frey procedures. There have also been reports of successful robotic Puestow procedures with Fernandes *et al*[21] reporting on eight patients who underwent the operation with resolution of pain in 80% of patients, and a 0% anastomotic fistula rate. Giulianotti *et al*[40] have reported a case of robotic assisted PD with preservation of the vascular supply for autologous islet cell transplantation. The authors reported dissection of the pancreas whilst maintaining arterial supply and venous drainage *via* the gastroduodenal artery and the superior pancreaticoduodenal vein[40]. Another group reported the first robotic total pancreatectomy with auto islet transplantation, again preserving blood supply until the final moments before specimen removal[41]. While these reports describe uncommon procedures and do not carry statistical weight, they do illustrate the versatility of the robotic platform in particular its utility in performing intricate vascular dissections.

DISCUSSION

The pancreas, in particular the head of the gland continues to be a relatively unexplored territory in terms of laparoscopic pancreatic resection. There are of course a few pioneers who have conquered the learning curve, but it is a challenge for the average surgeon to achieve. Robotic technology assists the surgeon in overcoming many of the obstacles that render the totally laparoscopic approach unfeasible. The superior visualization (Figure 1D) and restoration of hand-eye coordination along with the increased dexterity allowed by the robotic platform seems to empower even a moderate volume surgeon to complete complex pancreatic resection and reconstruction with at least equivalent results to the open approach with an acceptable learning curve. The minimally invasive approach as described above is associated with a reduc-

tion in blood loss, a reduced length of stay and perhaps a reduction in overall complications. There is however no difference in the rate of post-operative pancreatic fistula or mortality. Oncologic outcomes in selected patients appear equivalent and perhaps better than open surgery in terms of margin status and lymph node harvest but long-term outcomes are as yet unknown. Future studies should not only monitor long-term oncologic outcomes but also the outcomes of surgery such as adhesive bowel obstruction, incisional hernia formation and chronic pain. Such data will inform the risk to benefit profile of each individual operation, this is particularly important for patients undergoing pancreatic resection for pre-malignant disease *e.g.*, IPMN and indeed for those patients undergoing surgery for pancreatic cancer as improved systemic therapies lead to long term survival.

One may argue that robotic assistance may not improve outcomes beyond the standard minimally invasive approach, for example the use of the robot in laparoscopic colon and rectal surgery has not proven superior to standard laparoscopy[42]. This may be indeed be the case in DP, however, the use of the robot seems to make minimally invasive PD a safe and realistic option whereas many surgeons felt totally laparoscopic PD was not a feasible option with traditional laparoscopic instruments.

Robotic surgery is commonly criticized for the costs involved. Robotic prostatectomy was the first robotic operation to gain widespread acceptance across the surgical community, notwithstanding this several groups have reported that it is not cost effective despite reduced morbidity and length of stay[43,44]. There are sparse cost data available for robotic pancreatic surgery, however, two groups have reported that the higher costs associated with the robotic approach were offset by a reduction in the length of stay[34,45]. The cost of the robotic approach may also be offset by a quicker return to economic productivity by the patient and reduction in the long-term effects associated with laparotomy. Intuitive Surgical® holds a monopoly on robotic surgical technology but one can anticipate future competition, which will lead to a reduction in cost.

In summary, it is rational to conclude that robotic pancreatic surgery is feasible and safe in an environment where surgeons and support staff are appropriately trained. One must bear in mind that the published results originate from high volume specialist centers and may not apply to lower volume practitioners, furthermore

the possibilities of patient selection and publication bias are real. It is not clear whether the robotic approach affords the patient and society a net benefit both in terms of "cure" and overall cost. The natural solution to this impasse would be to perform a randomized control trial to define the clinical utility of the robotic approach *vs* traditional approaches. Like many technological innovations it is likely that the surgical community will adopt the technology before such robust evidence is furnished. Given the unlikelihood of a fully powered randomized controlled trial we must continue to scrutinize outcomes particularly long-term oncologic outcomes. In this changing healthcare environment it is likely that both government and third party payers will demand data that supports the superiority of new technologies such as the robot given the substantial equipment costs.

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2014 ADVANCES IN PANCREATIC CANCER

Adjuvant therapy in pancreatic cancer

Owain Peris Jones, James Daniel Melling, Paula Ghaneh

Owain Peris Jones, James Daniel Melling, Paula Ghaneh, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, 5th Floor UCD Building, Daulby Street, Liverpool L69 3GA, United Kingdom

Author contributions: All three authors contributed to the literature search, writing and editing of this manuscript.

Correspondence to: Paula Ghaneh, Professor, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, 5th Floor UCD Building, Daulby Street, Liverpool L69 3GA, United Kingdom. p.ghaneh@liverpool.ac.uk

Telephone: +44-151-7064170 **Fax:** +44-151-7065826

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Abstract

Pancreatic cancer remains one of the leading causes of cancer related death worldwide with an overall five-year survival of less than 5%. Potentially curative surgery, which alone can improve 5-year survival to 10%, is an option for only 10%-20% of patients at presentation owing to local invasion of the tumour or metastatic disease. Adjuvant chemotherapy has been shown to improve 5-year survival to 20%-25% but conflicting evidence remains with regards to chemoradiation. In this article we review the current evidence available from published randomised trials and discuss ongoing phase III trials in relation to adjuvant therapy in pancreatic cancer.

Key words: Pancreatic cancer; Adjuvant; Gemcitabine; Chemotherapy; Chemoradiotherapy; Phase III

Core tip: This paper discusses every major trial undertaken in the field of adjuvant therapy in pancreatic cancer. The evolution of chemotherapeutic regimes over the past 25 years and the controversy surrounding chemoradiation are analysed, in addition to looking at the phase III trials currently in progress.

INTRODUCTION

Despite accounting for only 2.2% of all cancers, pancreatic ductal adenocarcinoma is the fourth most common cause of cancer related death in the world[1]. In 2008 there were 279000 new cases worldwide with 266000 deaths from the disease, reflecting its dismal prognosis. Owing to the majority of patients presenting with locally advanced and metastatic disease, the overall survival rates at one and five years after diagnosis are 19% and 0.4%-4%[2] respectively. Surgery is the single most important factor in improving outcome but only 10%-20% of patients are candidates for such treatment which can improve the five-year survival rate to 10%[3]. This modest survival benefit is due to the high prevalence of both local recurrence and distant metastases due to residual microscopic disease. In recent years, interest has increased exponentially in both neoadjuvant and adjuvant strategies to improve these outcomes.

ADJUVANT CHEMOTHERAPY

A handful of chemotherapy regimens had been utilised in locally advanced and metastatic pancreatic cancer in the 1970s and 1980s with limited success. Response rates of 30%-43%[4] were reported and though these patients achieved some survival benefit, this evidence was not strong enough to recommend its routine use in all patients. Mallinson *et al*[5] was amongst the first to publish on the benefit of 5-flurouracil (5-FU) based chemotherapy in the palliative setting of pancreatic cancer, reporting a median survival of 44 wk in those receiving treatment against only 9 wk in controls. In 1993, a Norwegian

group[6] was first to publish a randomised study assessing the role of adjuvant chemotherapy in resected pancreatic cancer (Table 1). Sixty-one patients (47 pancreatic and 14 ampullary cancers) were divided into two treatment arms - one to undergo surgery alone and the second to undergo adjuvant chemotherapy. This adjuvant therapy consisted of 5-FU 500 mg/m², doxorubicin 40 mg/m² and mitomycin C 6 mg/m² every 3 wk for six cycles. Median survival was improved to 23 mo with adjuvant chemotherapy in comparison to 11 mo in those undergoing observation alone ($P = 0.04$). One-year survival improved to 70% with chemotherapy as opposed to 45% in the observation group but unfortunately this did not translate into a longer-term survival benefit. A potential explanation may be the high toxicity rate in the treatment group, which resulted in only 56% completing the prescribed chemotherapy course.

The landmark ESPAC-1[7] (European Study Group for Pancreatic Cancer) study was designed to determine whether adjuvant chemoradiotherapy or adjuvant chemotherapy alone had a role in improving survival following pancreatic cancer resection. This was the first adequately powered randomised trial to assess adjuvant therapy in pancreatic cancer, recruiting 541 patients over a six year period in 61 centres internationally. Inclusion criteria consisted of patients having made a full recovery from a macroscopically resected pancreatic ductal adenocarcinoma, with a life expectancy of over 3 mo. Two hundred and eighty five patients were randomised in a two-by-two factorial design to receive chemoradiotherapy alone, chemotherapy alone, both or observation. In addition to this 2 × 2 design, a further 256 patients were also randomised to receive either chemoradiotherapy, chemotherapy, or observation (Individual treatment groups). Chemotherapy consisted of a 20 mg/m² intravenous bolus of folinic acid, followed by a further intravenous bolus of 5-FU (425 mg/m²) to be administered on days 1-5 of a 28 d cycle, over 6 cycles.

With a median follow-up of 10 mo [range 0-62, interquartile range (IQR) 1-25] for surviving patients, initial results were suggestive of a significant improvement in outcome in those receiving chemotherapy when considering the entire study population. Median survival was 19.7 mo (95%CI: 16.4-22.4) in those receiving chemotherapy, against 14 mo (95%CI: 11.9-16.5) in those not receiving any [hazard ratio (HR) = 0.66, 95%CI: 0.52-0.83, $P = 0.0005$]. However, this significance was lost when analysing those patients in the 2 × 2 design alone (17.8 mo *vs* 15.8 mo, HR = 1.3, 95%CI: 0.96-1.77, $P = 0.09$).

The final analysis of the 2 × 2 ESPAC-1 data[8] was based on 237 deaths in 289 patients with a median follow up of 47 mo (IQR 33-62 mo). Median survival was 20.1 mo (95%CI: 16.5-22.7) amongst patients who had undergone chemotherapy *vs* 15.5 mo (95%CI: 13-17.7) in those who had not (HR = 0.71, 95%CI: 0.55-0.92, $P = 0.009$). The estimated two and five year survival was 40% *vs* 21% and 21% *vs* 8% respectively in those who received chemotherapy against patients which had not.

Exclusive to the 2 × 2 study design, the cohort of 75 patients that received chemotherapy alone fared significantly better than those who underwent observation ($n = 69$). Median survival was 21.6 mo (95%CI: 13.5-27.3) *vs* 16.9 mo (95%CI: 12.3-24.8) and estimated five-year survival was 29% *vs* 11%. ESPAC-1 established 5-FU and folinic acid as the drug of choice in the adjuvant treatment of pancreatic cancer.

Takada *et al*[9] recruited a total of 508 patients with various resected pancreaticobiliary cancers which included 173 pancreatic malignancies. Though this multicentre randomised controlled trial recruited patients between 1986 and 1992, it was published later than ESPAC-1, in 2002. Patients were randomised in this study to receive either adjuvant mitomycin C (rapid intravenous infusion of 6 mg/m² on the day of surgery) and 5-FU (310 mg/m² for days 1-5 of postoperative weeks 1 and 3, followed by a daily dose of 100 mg/m² from week 5 until disease recurrence) or surgery alone. In the pancreatic subset of patients, this chemotherapy regime showed no significant improvement in 5-year survival or 5-year disease free survival. Unusually, this study utilised oral 5-FU as opposed to the usual intravenous form which may offer a reason for its ineffectiveness.

Kosuge *et al* (JSAP)[10] published a randomised trial evaluating adjuvant cisplatin (80 mg/m² on day 1) and 5-FU (continuous infusion of 500 mg/m² on days 1-5) with a second cycle of chemotherapy 4-8 wk after the first. As only those having undergone a R0 resection for ductal pancreatic cancer were included, only 89 patients were recruited over 8 years, resulting in an underpowered study. No significant difference was identified in median survival, 5-year survival and 5-year disease-free survival in comparison to patients undergoing surgery alone. It must be mentioned that approximately two-thirds of these patients also underwent 30 Gy of intraoperative radiotherapy on a non-randomised basis, but this therapy proved insignificant as a prognostic indicator in a multivariate analysis.

In 1997, Burris *et al*[11] published their randomised control trial comparing the nucleoside analogue gemcitabine with 5-FU in advanced pancreatic cancer. In addition to demonstrating a clinical benefit with regards to pain relief, weight and performance status, those receiving gemcitabine achieved a one-year survival rate of 18% as opposed to 2% with 5-FU. These findings resulted in the recruitment of patients for CONKO-001[12] between 1998 and 2004. This randomised study compared adjuvant gemcitabine (six cycles of a 30 min intravenous infusion at 1000 mg/m² during weeks 1-3 followed by a break at week 4) with observation alone in patients undergoing a curative pancreatic cancer resection. In addition to clinical follow up and serum biochemistry checks, two-monthly ultrasound scans were performed to assess any recurrence. A computed tomography (CT) scan was also performed at the termination of chemotherapy (gemcitabine group) or at six months (observation group). Three hundred and sixty eight patients were re-

Table 1 Major adjuvant chemotherapy trials in pancreatic cancer

Year published	Author/group	Treatment arms (n)	Final analysis			Survival (95%CI)					Median DFS (mo)	Disease-free survival (DFS)(95%CI)			
			T3	N+	R0	Median survival (mo)	1-yr survival	2-yr survival	3-yr survival	5-yr survival		1-yr DFS	2-yr DFS	3-yr DFS	5-yr DFS
1993	Bakkevold	5-FU/doxorubicin/mitomycinc (30)	NA	NA	100%	23	-	70%	27%	4%	-	-	-	-	-
		Surgery alone (31)				11 (*P* = 0.02)		45%	30%	8% (*P* = 0.1)					
2001	ESPAC-1 (all patients)	5-FU/folinic fcid +/- CRT (238)	NA	53%	82%	19.7 (16.4-22.4)	-	-	-	-	-	-	-	-	-
		No chemotherapy +/- CRT (235)				14 (11.9-16.5) (HR = 0.66, 0.52-0.83, *P* = 0.0005)									
	ESPAC-1 (2 × 2 design only)	5-FU/folinic fcid +/- CRT (146)	NA	NA	NA	17.4 (13.5-21.8)	-	-	-	-	-	-	-	-	-
		No chemotherapy +/- CRT (139)				15.9 (13.5-19.2) (HR = 0.82, 0.6-1.11, *P* = 0.19)									
2002	Takada	5-FU/mitomycin C (89)	NA	85%	58%	NA	-	-	-	11.5%	-	-	-	-	8.6%
		Surgery alone (84)								18% (log rank NS)					7.8% (log rank *P* = 0.84)
2004	ESPAC-1 (2 × 2 final analysis)	5-FU/folinic fcid (147)	NA	54%	82%	20.1 (16.5-22.7)	-	40%	-	21%	-	-	-	-	-
		No chemotherapy +/- CRT (142)				15.5 (13-17.7) (HR = 0.71, 0.55-0.92, *P* = 0.009)		30%		8%					
2006	JSAP (Kosuge)	Cisplatin/5-FU (45)	NA	27%	100%	12.5	-	-	-	26.4%	-	-	-	-	-
		Surgery alone (44)				15.8				14.9% (*P* = 0.94)					
2007	CONKO-001 (Oettle)	Gemcitabine (179)				22.1 (18.4-25.8)	72.5%	47.5%	34%	22.5%	13.4 (11.4-15.3) (*P* < 0.001)	58%	30.5%	23.5%	16.5%
		Surgery alone (175)	86	72%	83%	20.2 (17-23.4) (*P* = 0.06)	72.5%	42%	20.5%	20.5%	6.9 (6.1-7.8) (*P* < 0.001)	31%	14.5%	7.5%	5.5%
2008	CONKO-001 Final (Neuhaus)	Gemcitabine (179)				22.8	-	-	36.5	21	13.4	-	-	23.5	16.0

Year	Study	Treatment (n)				Median OS (mo)					Median DFS (mo)				
		Surgery alone (175)				20.2 (P = 0.005)			19.5%	9%	6.9 (P < 0.001)			8.5%	6.5%
2009	JSAP-2 (Ueno)	Gemcitabine +/- RT (58)	86%	69%	84%	22.3 (16.1-30.7)	77.6%	48.3%	-	23.9%	11.4 (8-14.5)	49%	27.2%	-	-
		Surgery alone +/- RT (60)	75%			18.4 (15.1-25.3) (HR = 0.77, 0.51-1.14, P = 0.19)	75%	40%	-	10.6%	5 (3.7-8.9) (HR = 0.6, 0.4-0.89, P = 0.01)	26.7%	16.7%	-	-
2009	Collated data ESPAC-1, ESPAC-1 plus, ESPAC-3(v1)	5-FU/folinic fcid (233)	NA	55%	75%	23.2 (20.1-26.5)	77%	49%	-	24%	-	-	-	-	-
		Surgery alone (225)	63%			16.8 (14.3-19.2) (HR = 0.7, 0.55-0.88, P = 0.003)	63%	37%	-	14%	-	-	-	-	-
2010	ESPAC-3(v2)	5-FU/folinic fcid (551)	NA	72%	65%	23 (21.1-25)	78.5% (75%-82%)	48.1% (43.8%-52.4%)	-	-	14.1 (12.5-15.3)	56.1% (51.8%-60.3%)	30.7% (26.7%-34.6%)	-	-
		Gemcitabine (537)				23.6 (21.4-26.4) (HR = 0.94, 0.81-1.08, P = 0.39)	80.1% (76.7%-83.6%)	49.1% (44.8%-53.4%)	-	-	14.3 (13.5-15.6)	61.3% (57.1%-65.5%)	29.6% (25.6%-33.5%)	-	-
2013	JASPAC-01	S-1 (187)	87%	63%	87%	46.3	70% (HR = 0.56, 0.42-0.74, P < 0.0001)		-	-	23.2	49% (HR = 0.56, 0.43-0.71, log rank P < 0.0001)		-	-
		Gemcitabine (191)				25.5 (P < 0.0001)			-	-	11.2 (log rank P < 0.0001)		29% (HR = 0.56, 0.43-0.71, log rank P < 0.0001)	-	-

CRT: Chemoradiotherapy; NA: Not avialable; NS: Not significant; 5-FU: 5-flurouracil.

cruited with a median follow-up of 53 mo. The main outcome of this study was that gemcitabine significantly improved disease-free survival following pancreatic cancer resection. Median disease-free survival in the gemcitabine group was 13.4 mo (95%CI: 11.4-15.3) as opposed to only 6.9 mo (95%CI: 6.1-7.8) in the observation group (P < 0.001). Importantly, this significant disease-free survival benefit was maintained whether patients had undergone an R0 or R1 resection. With regards median overall survival, the gemcitabine benefit was only marginal in comparison to the observation group (22.8 mo vs 20.2 mo, P = 0.005)[13]. This minimal difference is potentially explained by the authors by the fact that almost all patients that relapsed in the observation group received gemcitabine or a further line of chemotherapy. However, this study established gemcitabine as the favoured adjuvant chemotherapeutic agent particularly due to its excellent toxicity profile in comparison to 5-FU.

Coincidentally, at the same time as CONKO-001, a further study to compare adjuvant gemcitabine and observation was being undertaken. One hundred and nineteen patients were recruited between 2002 and 2005 for JSAP-2[14], with a median follow up for surviving patients of over five years. Gemcitabine resulted in a median disease-free survival of 11.4 mo (95%CI: 8-14.5) against 5 mo (95%CI: 3.7-8.9) in the observation group (HR = 0.60, 95%CI: 0.40-0.89, P = 0.01). This however did not convert to a significant benefit in overall survival as the study was underpowered. Another factor may be that though this study utilised an identical gemcitabine dosage regimen as used in CONKO-001, only three cycles were administered as opposed to the six used in the European study. It must also be noted that the median number of days between surgery and randomisation was nearly double that of CONKO-001. Just over half of patients in this study (52%) also received intraoperative radiotherapy, though the authors argue that this effect may have been negligible.

Following on from the ESPAC-1 study, the group undertook another randomised controlled trial, ESPAC-3 to compare 5-FU and folinic acid (as per the ESPAC-1 regime),

Table 2 Meta-analyses of adjuvant chemotherapy in pancreatic cancer

Year published	Author	Arm (*n*)	Survival (95%CI)		
			Median survival (mo)	2-yr survival	5-yr survival
2005	Stocken *et al*[20]	CT (348)	19 (16.4-21.1)	38%	19%
		No CT (338)	13.5 (12.2-15.8)	28%	12%
2007	Boeck *et al*[24]	CT (482)	3 mo (0.3-5.7) survival benefit	-	3.1% (-4.6%-10.8%) survival benefit
		No CT (469)	with CT *vs* no CT (*P* = 0.03)		with CT *vs* no CT (*P* > 0.05)
2008	Butturini *et al*[25]		R0 resections		
		CT (236)	20.8 (17.7-23.2)	42% (35%-48%)	22% (17%-28%)
		No CT (222)	13.8 (12.2-16.4)	27% (21%-33%)	10% (5%-14%)
			R1 resections		
		CT (109)	15 (11.7-18.1)	29% (20%-38%)	14% (7%-21%)
		No CT (114)	13.2 (10.5-17.6)	31% (22%-40%)	17% (10%-24%)
2013	Liao *et al*[26]		Hazard ratio for death (95%CI)		
		Flurouracil (876)	0.62 (0.42-0.88)		
		Observation (670)			
		Gemcitabine (774)	0.68 (0.44-1.07)		
		Observation (670)			
		Gemcitabine (774)	1.1 (0.70-1.86)		
		Flurouracil (876)			

CT: Chemotherapy.

gemcitabine (as per CONKO-001 regime) and surgery alone in resected pancreatic cancer. During recruitment however, the publication of ESPAC-1 proved the undoubted benefit of adjuvant chemotherapy. This resulted in the observation arm being forfeited and the study was renamed ESPAC-3(v2)[15].

ESPAC-3(v2) was the largest study of its kind, recruiting 1088 patients with pancreatic ductal adenocarcinoma in 159 centres worldwide over a seven year period. With a median follow-up of 34.2 mo (range: 0.4-86.3 mo, IQR 27.1-43.4), no significant difference was shown in overall survival or progression-free survival between the two treatment groups. However, gemcitabine halved the number of serious treatment-related adverse events compared to its opposing arm (14% *vs* 7.5% of patients, *P* < 0.001). It was also noted a more favourable outcome was achieved in patients with node positive disease or an R1 resection when administered gemcitabine. This firmly established the drug as the current gold standard in the adjuvant treatment of pancreatic cancer.

A meta-analysis in 2009[16] combined data from a subgroup of ESPAC-3(v1) with ESPAC-1 2 × 2 and ESPAC-1 Plus (a subgroup of 192 patients in a randomised comparison between 5-FU and observation +/- chemoradiation). The purpose of this publication was to ascertain the benefit of adjuvant 5-FU and folinic acid (*n* = 233) as opposed to surgery alone (*n* = 225). Median survival was 23.2 mo (95%CI: 20.1-26.5) in the chemotherapy group, in comparison to 16.8 mo (95%CI: 14.3-19.2) in those patients in the observation arm (HR = 0.7, 95%CI: 0.55-0.88, *P* = 0.003). Chemotherapy also improved overall survival at one, two and five years, providing robust evidence for the continued use of 5-FU and folinic acid in the adjuvant setting alongside gemcitabine.

The most recently published randomised controlled trial, JASPAC-01[17] enrolled 385 patients between 2007 and 2010 to compare adjuvant gemcitabine with fluorinated pyrimidine S-1 in resected pancreatic cancer. The gemcitabine regime was identical to CONKO-001 with an S-1 regime of four cycles of 80 mg/m² per day for four weeks, followed by a fortnight rest. Promising interim results were presented in 2012[18] with an overall 2-year survival of 53% and 70% in the gemcitabine and S-1 groups respectively (HR = 0.56, 95%CI: 0.42-0.74, *P* < 0.0001). S-1 also proved superior with regards to recurrence-free survival at 2-years with 49% of patients remaining disease-free compared with 29% in the gemcitabine cohort (HR = 0.56, 95%CI: 0.43-0.71, log-rank *P* < 0.0001). S-1's comparatively low toxicity in addition to the fact that S-1 is orally administered, would be partly responsible for the superior quality of life scores in this group (*P* < 0.0001). Though the authors concluded that S-1 should be considered the new standard treatment for resected pancreatic cancer, there is doubt whether this agent will ever be of broad benefit in the West. It has been stated that due to the metabolic differences between Asian and Caucasian populations, gastrointestinal side effects are far greater in the latter leading to lower tolerated doses of S-1[19].

META-ANALYSIS OF ADJUVANT CHEMOTHERAPY IN PANCREATIC CANCER

The first meta-analysis assessing adjuvant therapy in pancreatic cancer was published in 2005 (Table 2). Stocken *et al*[20] included five randomised trials [Bakkevold *et al*[6], ESPAC-1[7], Takada *et al*[9], EORTC[21] and Gastrointestinal Study Group (GITSG)[22,23] to evaluate the effects of both chemotherapy and chemoradiotherapy in the adjuvant setting (*n* = 939). With the exception of GITSG, the authors collected individual patient data from each

of these studies (n = 875) to produce as accurate a results as possible. When collating results from the three chemotherapy trials, heterogeneity was affected with the addition of the Japanese results (χ^2 = 11.7, P = 0.009 when included, χ^2 = 2.5, P = 0.29 without). The authors suggest that this was due to the large number of R1 resections included in that particular study. Nevertheless, analysis of the dataset both including and excluding this study resulted in reductions of 25% (HR = 0.75, 95%CI: 0.64-0.9, P = 0.001) and 35% (HR = 0.65, 95%CI: 0.54-0.8, P < 0.001) respectively in the risk of death with adjuvant chemotherapy. Median survival was estimated to be 19 mo (95%CI: 16.4-21.1) with chemotherapy and 13.5 mo (95%CI: 12.2-15.8) without.

A later meta-analysis[24] included the five randomised trials comparing adjuvant chemotherapy to observation (Bakkevold *et al*[6], ESPAC-1[7], Takada *et al*[9], JSAP[10] and CONKO-001[12]). Median survival data was available from all studies with the exception of Takada *et al*[9], with no significant heterogeneity between the remaining four conflicting studies (P = 0.07). Meta-analysis indicated a significant survival benefit of 3 mo (95%CI: 0.3-5.7, P = 0.03) in patients receiving adjuvant chemotherapy as opposed to observation. However, adjuvant treatment translated into only a 3.1% benefit in 5-year survival which proved insignificant.

A third meta-analysis looked specifically at adjuvant therapy in relation to resection margins[25]. This meta-analysis was supportive of adjuvant chemotherapy, indicating a 25% reduction in the risk of death with treatment as opposed to observation (HR = 0.75, 95%CI: 0.64-0.9, P = 0.001). Patients undergoing a clear-margin resection benefited from a 7-mo survival increase with chemotherapy (median survival of 20.8 mo *vs* 13.8 mo), but the effect was less pronounced in R1 resections (median survival of 15 mo *vs* 13.2 mo). This finding was in agreement with Stocken who noted that chemotherapy was less effective in patients with a positive resection margin.

A recently published network meta-analysis[26] has examined overall survival in patients receiving adjuvant gemcitabine or 5-FU in comparison to observation. Results suggested that adjuvant therapy with either gemcitabine (n = 774) or 5-FU (n = 876) showed a survival benefit in comparison with observation alone (n = 670) with hazard ratios of 0.68 (95%CI: 0.44-1.07) and 0.62 (95%CI: 0.42-0.88) respectively. No significant survival difference was noted in comparing adjuvant gemcitabine and 5-FU, though grades 3-4 non-haematological toxicity was almost four-times as common in patients receiving the latter drug.

ADJUVANT CHEMORADIOTHERAPY

GITSG[22] was the first randomised trial evaluating the role of adjuvant therapy in pancreatic cancer (Table 3). In non-resectable patients, previous studies had shown the benefit of both radiotherapy[27] and 5-FU combined with radiotherapy[28] and on this basis GITSG compared

adjuvant 5-FU chemoradiation *vs* no adjuvant therapy. Though the study population was small (n = 43), final analysis of the data revealed a substantial median survival benefit with treatment (21 mo) in comparison to no treatment (10.9 mo). Following this evidence, chemoradiotherapy became a standard adjuvant treatment option for pancreatic cancer patients in the United States[29]. A decade later, the findings from GITSG were supported by a prospective, non-randomised study. Yeo *et al*[30] offered two different chemoradiotherapy regimes (standard or intensive) or observation. Patients undergoing adjuvant treatment reported a median and one-year survival of 19.5 mo and 80%, in comparison to 13.5 mo and 54% in those undergoing observation (P = 0.003). Multivariate analysis also supported a survival benefit to those receiving either standard (P < 0.001) or intensive therapy (P = 0.04).

The EORTC study[21] was undertaken across twenty nine European centres and included 218 patients who had undergone resection for pancreatic or ampullary lesions. One hundred and fourteen of these were for pancreatic head cancers and those tumours graded as T3 $\leqslant$ or N1b nodal disease were excluded from the study. Patients were assigned surgery alone or to additionally receive two four-week cycles of adjuvant 5-FU and concurrent radiotherapy. Treatment was commenced within eight weeks of surgery when patients received a daily radiotherapy dose of 2 Gy, five times a week for two weeks followed by a two week break. This cycle was then repeated to make a total absorbed dose of 40 Gy. Alongside the first week of radiotherapy, patients received 25 mg/kg of 5-FU per 24 h up to a maximum daily dose of 1500 mg. The dosage of 5-FU during the second cycle was dependant on any resulting toxicity from the first cycle.

Analysis of the entire study population revealed no statistical difference in overall or disease-free survival. When considering the pancreatic group alone, median survival in the treatment group was 17.1 mo compared to 12.6 mo in those undergoing observation (P = 0.099). Two-year survival was 37% and 20%, with five-year survival being 23% and 10% in each group respectively. The pattern of recurrent disease was similar in both treatment groups with both locoregional and distant metastases occurring concurrently in 19%-22% of patients experiencing disease recurrence. 15% of patients from each group experienced local recurrence alone, suggesting that adjuvant radiotherapy is ineffective against pancreatic and ampullary cancer.

In the ESPAC-1 trial, chemoradiotherapy consisted of 20 Gy in ten daily fractions over a fortnight with a 500 mg/m² intravenous bolus of 5-FU on days 1-3 to be repeated two weeks later. For those patients assigned to receive both chemoradiotherapy and chemotherapy, the above regime was combined with the chemotherapy regime previously described. Initial results from ESPAC-1 showed no survival benefit in those receiving chemoradiotherapy. Chemoradiotherapy incurred a median survival of 15.5 mo (95%CI: 13.5-17.4) compared to

Table 3 Major adjuvant chemoradiotherapy trials in pancreatic cancer

Year published	Author/group	Treatment arms (n)	Final analysis			Median survival (mo)	Survival (95%CI)			Disease-free survival (DFS) (95%CI)	
			T3	N+	R0		2-yr survival	3-yr survival	5-yr survival	Median DFS (mo)	2-yr DFS
1985	GITSG	CRT (21)	37	28	100	21	43% (0.25%-0.63%)	-	-	-	-
		Surgery alone (22)				10.9	18% (0.08%-0.36%)				
1999	EORTC	5-FU/RT (104)	21	46	77	24.5	51% (41%-61%)	-	28% (17%-39%)	17.4	38% (28%-48%)
		Surgery alone (103)				19 (log rank P = 0.208)	41% (31%-51%)		22% (12%-32%)	16 (P = 0.643)	37% (27%-47%) (P = 0.643)
		5-FU/RT (60)	0	51	NA	17.1	37% (24%-50%)	-	20% (5%-35%)	-	-
		Surgery alone (54)				12.6 (log rank P = 0.099)	23% (11%-35%)		10% (0%-20%)		
2001	ESPAC-1 (all patients)	CRT +/- 5-FU/folinic acid (175)	NA	56	82	15.5 (13.5-17.4)	-	-	-	-	-
		No CRT +/- 5-FU/folinic acid (178)				16.1 (13.1-20.1) (HR = 1.18, 0.9-1.55, P = 0.24)					
	ESPAC-1 (2 × 2 design only)	CRT +/- 5-FU/folinic acid (142)	NA	NA	NA	15.8 (13.5-19.4)	-	-	-	-	-
		No CRT +/- 5-FU/folinic acid (143)				17.8 (14-23.6) (HR = 1.3, 0.96-1.77, P = 0.09)					
2004	ESPAC-1 (2 × 2 final analysis)	CRT +/- 5-FU/folinic acid (145)	NA	53	82	15.9 (13.7-19.9)	29%	-	10%	-	-
		No CRT +/- 5-FU/folinic acid (144)				17.9 (14.8-23.6) (HR = 1.28, 0.99-1.66, P = 0.05)	41%		20%		
	ESPAC-1 (Individual Treatment Groups)	5-FU/folinic acid (75)				21.6 (13.5-27.3)	-	-	29%	-	-
		CRT + 5-FU/folinic acid (72)				19.9 (14.2-22.5)			13%		
		Observation (69)				16.9 (12.3-24.8)			11%		
		CRT (73)				13.9 (12.2-17.3)			7%		
2006	RTOG 97-04	CRT + 5-FU (230)	75	66	66	No significant difference	-	22%	-	-	-
		CRT + gemcitabine (221)						31%			
		CRT + 5-FU (201)	NA	NA	NA	16.9					
		CRT + gemcitabine (187)				20.5 (HR = 0.82, 0.65-1.03, P = 0.09)					
2011	RTOG 97-04 (5-yr analysis)	CRT + 5-FU (230)	75	66	66	No significant difference	35%	23%	19%	n/s	-
		CRT + gemcitabine (221)				HR = 0.933, 0.76-1.145, P = 0.51	40%	27%	19%		
		CRT + 5-FU (201)	NA	NA	NA	17.1	34%	21%	18% (13%-24%)	-	
		CRT + gemcitabine (187)				20.5	42%	28%	22%		
2012	CapRI (Schmidt)	5-FU/cisplatin/interferon α-2b → RT → 5-FU (53)	97	79	61	32.1 (22.8-42.2)	-	-	-	15.2 (10.3-24.8)	-
		5-FU/folinic acid (57)				28.5 (19.5-38.6) (HR = 1.2, 0.49-2.95, P = 0.49)				11.5 (9.8-17.6) (P = 0.61)	

CRT: Chemoradiotherapy; NA: Not avaiable; 5-FU: 5-flurouracil.

16.1 mo (95%CI: 13.1-20.1) in patients which had not received any (HR = 1.18, 95%CI: 0.9-1.55, P = 0.235). These findings were echoed when evaluating patients in the 2 × 2 study design alone with those patients receiving chemoradiotherapy alone achieving a median survival of 15.8 mo (95%CI: 13.5-19.4) *vs* 17.8 mo (95%CI: 14-23.6) in those not. Again, these findings did not reach statistical significance with P = 0.086.

Final analysis of the 2 × 2 data showed that chemoradiotherapy had a negative effect on patient survival. Median survival was 15.9 mo (95%CI: 13.7-19.9) in those that received chemotherapy whilst patients that received none survived for a median of 17.9 mo (95%CI: 14.8-23.6), $P = 0.05$. Estimated 5-year survival was 10% in the chemoradiotherapy cohort in comparison to 20% in those patients who received none. In those patients randomised outside the 2 × 2 study design, median survival was only 13.9 mo (95%CI: 12.2-17.3) amongst the 73 patients who had received chemoradiotherapy. This was in comparison to a median survival of 16.9 mo (95%CI: 12.3-24.8) in those who underwent surgery alone and 21.6 mo (95%CI: 13.5-27.3) in those who underwent adjuvant chemotherapy without any chemoradiation. Estimated 5-year survival in these individual treatment groups was 7%, 11% and 29% respectively. Though this is strongly suggestive that adjuvant chemoradiation has a negative impact on survival, ESPAC-1 was underpowered to directly assess these smaller cohorts outside the 2 × 2 design. The authors suggest that the lack of survival benefit with chemoradiation may be due to a delay in administering the treatment to patients who were also receiving chemotherapy. Some have argued that the radiotherapy given during ESPAC-1 was substandard and not subject to rigorous quality control, though the survival rates achieved in the individual groups were similar to those achieved in other major studies[31].

Following the publication of their interim findings in 2008[32], the final 5-year analysis of the RTOG 97-04 study was published in 2011[33]. Patients were stratified into two arms - each to receive either gemcitabine or 5-FU both prior to, and after 5-FU based chemoradiation. In the gemcitabine group ($n = 221$), one cycle was administered (1000 mg/m² for 3 wk followed by a 1 wk break) prior to chemoradiotherapy, after which a further 3 cycles was undertaken. Patients in the 5-FU arm ($n = 230$) were administered a continuous infusion of 250 mg/m² per day for 3 wk prior to the commencement of chemoradiation. 5-FU was then administered for two six-week cycles of 5-FU (4 wk on plus 2 wk off). Chemoradiation consisted of 50.4 Gy of radiation in divided fractions, in association with a continuous infusion of 5-FU at the previously stated dose over the duration of the radiation therapy. No significant difference was identified in overall or disease-free survival in the final analysis. Worth noting, is though completion rates for designated treatments were equally high (87% in the 5-FU group and 90% in the gemcitabine group) those in the latter group experienced greater numbers of haematological ($P < 0.001$) and Grade 4 events ($P < 0.001$) secondary to acute toxicity.

A subgroup analysis from RTOG was undertaken observing those with pancreatic head tumours. The difference in median survival between both groups was not statistically significant, being 17.1 mo in the 5-FU group and 20.5 mo in the gemcitabine group (HR = 0.933, 95%CI: 0.76-1.15, log rank $P = 0.51$). However, following adjustment for stratification variables including nodal status, tumour size and surgical margins, multivariate analysis suggests a benefit with gemcitabine over 5-FU (HR = 0.80, 95%CI: 0.63-1.00, $P = 0.05$).

The most recently published phase Ⅲ randomised trial compared adjuvant chemoradiation, including 5-FU, cisplatin and interferon Alfa-2b (Group 1) with adjuvant 5-FU and folinic acid without chemoradiation (Group 2). CapRI[34] followed a similar phase Ⅱ trial which reported a promising 5-year survival rate of 55%[35]. Patients in group 1 received a 200 mg/m² continuous infusion of 5-FU, 30 mg/m²/wk of cisplatin and 3 million units of interferon α-2b three times a week. This treatment continued for 5.5 wk and was given alongside 50.4 Gy of radiation in 28 fractions. Following chemoradiotherapy, patients underwent a further two cycles of 5-FU. Those in group 2 were treated for six cycles of chemotherapy with 20 mg/m² of folinic acid and 425 mg/m² of FU on days 1-5 of a 28-d cycle without any radiotherapy. Patients underwent regular clinical follow-up in addition to receiving a CT at 6-monthly intervals, or whenever clinically indicated. Though 132 patients were initially randomised, only 110 of these received at least one dose of a study treatment and were described as the per-protocol population.

Overall survival and disease free survival was not significantly different between the two groups. However, the median survival data from the per-protocol population are amongst the best published, being 32.1 mo (95%CI: 22.8-42.2) in group 1, and 28.5 mo (95%CI: 19.5-38.6) in group 2 (HR = 1.2, 95%CI: 0.49-2.95, $P = 0.49$). Selection bias was unlikely, given that 97% of tumours were T3 and above, 79% of patients had nodal disease and only 61% of patients underwent a R0 resection. The authors concede that these impressive survival figures are unlikely to be secondary to adjuvant therapy alone, and acknowledge that the vast majority of patients underwent an aggressive soft tissue clearance during their resection in Heidelberg. Nevertheless, these results seem to have been achieved at the expense of very high levels of toxicity, with 85% of patients receiving chemoradioimmunotherapy experiencing grades 3 or 4 toxicity which were mainly haematological in origin. In a separate study[36] this controversial chemoradioimmunotherapy regime led to a 93% grade 3 and 4 gastrointestinal toxicity rate, leading to its abandonment.

In addition to these randomised trials which have produced conflicting results, a handful of large retrospective reviews have also been published[37]. A prospectively collected database from John Hopkins Hospital in Baltimore compared those who received adjuvant chemoradiotherapy ($n = 271$) to those who did not ($n = 345$). Chemoradiotherapy consisted of a continuous infusion of 5-FU with 50 Gy of radiation in divided fractions, followed by maintenance 5-FU for a further 2-6 mo. Chemoradiation improved survival compared to those who received no adjuvant therapy, with a median survival of 21.2 mo as opposed to 14.4 mo (HR = 0.72, 95%CI: 0.6-0.86, $P < 0.001$). Even after adjusting for confounding factors in-

Table 4 Meta-analyses of adjuvant chemoradiotherapy in pancreatic cancer

Year published	Author	Arm (*n*)	Survival (95%CI)		
			Median survival (mo)	2-yr survival	5-year survival
2005	Stocken *et al*[20]	CRT	15.8 (13.9-18.1)	30%	12%
		No CRT	15.2 (13.1-18.2)	34%	17%
2008	Butturini *et al*[25]	R0 Resections			
		CRT (188)	15.9 (14-18.5)	30% (23%-36%)	10% (5%-15%)
		No CRT (183)	15.8 (13.4-20.1)	38% (31%-45%)	20% (13%-26%)
		R1 Resections			
		CRT (53)	14.7 (11.5-20.5)	30% (17%-42%)	18% (7%-29%)
		No CRT (53)	11.2 (9.4-16.7)	19% (8%-31%)	8% (0%-16%)
2013	Liao *et al*[26]		Hazard ratio for death (95%CI)		
		Chemoradiation (169)	0.91 (0.55-1.46)		
		Observation (670)			
		Chemoradiation + 5-FU (323)	0.87 (0.27-2.69)		
		5-FU (876)			
		Chemoradiation + 5-FU (323)	0.59 (0.19-1.74)		
		Chemoradiation (169)			
		Chemoradiation + gemcitabine (221)	0.82 (0.4-1.71)		
		Chemoradiation + 5-FU (323)			

CRT: Chemoradiotherapy; 5-FU: 5-flurouracil.

cluding comorbid disease and surgical complication rate amongst others, chemoradiation continued to show a survival benefit (relative risk = 0.74, $P < 0.001$). Multivariate analysis revealed a significant survival benefit to chemoradiotherapy in those patients who had either positive ($P = 0.002$) or negative ($P = 0.035$) resection margins.

The Mayo Clinic published their 1975-2005 experience[38], also supporting the use of adjuvant chemoradiotherapy. Their retrospective study ($n = 454$) included only R0 resections. Ninety-eight percent of the 274 patients that received radiotherapy (median dose 50 Gy in 28 fractions) also received concurrent 5-FU based chemotherapy, and only 10% of these received any additional chemotherapy following chemoradiation. Median survival was improved with adjuvant treatment as opposed to surgery alone, with rates of 25.2 and 19.2 mo respectively. Chemoradiotherapy improved survival in various disease subgroups including node positive disease ($P < 0.001$), high-grade tumours ($P < 0.001$), or both together ($P < 0.001$).

Numerous publications have recently originated from the US-based Surveillance, Epidemiology and End Results (SEER) database supporting the use of adjuvant radiotherapy. Artinyan *et al*[39] analysed 1930 patients that had undergone curative node-negative resections for pancreatic cancer during 1988-2003. Multivariate analysis revealed adjuvant radiotherapy to be a significant factor in improving overall survival (HR = 0.72, 95%CI: 0.63-0.82, $P < 0.001$). Greco *et al*[40] presented 2636 pancreatic resections, of which 1123 received adjuvant radiotherapy with a median survival of 18 mo, *vs* 11 mo in those who received no radiotherapy ($P < 0.01$). Moody *et al*[41] further supported these claims with his series, also concluding that adjuvant radiotherapy improved survival compared to no radiotherapy ($P = 0.004$). However, on subgroup analysis statistical significance was only maintained in patients with Stage 2B disease ($P < 0.0001$).

Hazard *et al*[42] also supports the use of radiotherapy with her publication, though specific conclusions in relation to adjuvant therapy cannot be made as most patients received both neoadjuvant and adjuvant radiation. Though the SEER database has the advantage of possessing the details of an impressive volume of patients, concerns have been raised. As the data collection is retrospective, now-important prognostic information such as margin status, lymphovascular and perineural invasion, patient comorbid status and performance status details have not been collected. This may have produced a treatment bias in these patient cohorts which cannot be adjusted for during statistical analysis.

META-ANALYSIS OF ADJUVANT CHEMORADIOTHERAPY IN PANCREATIC CANCER

In addition to the analysis of adjuvant chemotherapy data, Stocken *et al*[20] also pooled individual patient data from ESPAC-1 2×2[8], ESPAC-1 Plus[16] and EORTC[21] to assess the benefit of adjuvant chemoradiotherapy (Table 4). Despite borderline heterogeneity ($\chi^2 = 6.1$, $P = 0.05$), no significant difference in the risk of death was observed with chemoradiotherapy (HR = 1.09, 95%CI: 0.89-1.32, $P = 0.43$). The GITSG trial[22] was unfortunately unable to provide individual patient data and therefore summary data was utilised. Though heterogeneity was increased ($\chi^2 = 10$, $P = 0.02$) by the addition of the GITSG summary data to the individual data from other studies, the pooled HR again showed no difference in the risk of death between those receiving chemoradiotherapy and those not (HR = 1.02, 95%CI: 0.85-1.24, $P = 0.81$).

Butturini's meta-analysis[25] on adjuvant therapy and resection margins noted no significant survival advantage with chemoradiation in R0 resections (median survival

Table 5 Current phase III trials investigating adjuvant therapy in pancreatic cancer

Trial number	Co-ordinating country	First enrolment	Target sample size (n)	Adjuvant treatment arms	Primary outcome	Secondary outcomes (clinical only)
ISRCTN96397434 (ESPAC-4)	United Kingdom	2008	1396	(I) Gemcitabine (II) Gemcitabine plus capecitabine	OS	Toxicity Quality of life OS at 2 and 5 yr DFS at 5 yr
DRKS00000247 (CONKO-005)	Germany	2008	436	(I) Gemcitabine (II) Gemcitabine plus erlotinib	DFS	OS Toxicity
NCT01013649 (RTOG 0848)	United States	2009	950	(I) Gemcitabine (II) Gemcitabine plus erlotinib If DFS at end of treatment (I) or (II), further randomisation to: (III) A further course of (I) or (II) as previously received plus Capcitabine CRT (IV) A further course of (I) or (II) as previously received plus 5-FU CRT	OS	DFS Toxicity Correlation between baseline fatigue and survival
NCT01072981	United States	2010	722	Gemcitabine +/- 5-FU CRT +/- HyperAcute®-Pancreas (algenpantucel-L) immunotherapy	OS	
NCT01526135	France/Canada	2012	490	(I) Gemcitabine (II) mFolfirinox (5-FU, folinic acid, irinotecan, oxaliplatin)	DFS at 3 yr	OS at 3 yr
NCT01077427	Germany	2012	336	(I) Gemcitabine (II) Gemcitabine plus cisplatin plus regional hyperthermia	DFS	OS
NCT01964430	United States	Not yet active	-	(I) Gemcitaine (II) Gemcitabine plus nab-paclitaxel	DFS/OS	

OS: Overall survival; DFS: Disease-free survival; CRT: Chemoradiotherapy; 5-FU: 5-flurouracil.

15.8 and 15.9 mo). Though remaining statistically insignificant, there was evidence of a small survival benefit with adjuvant chemoradiation in patients receiving a R1 resection (median survival 11.2 and 14.7 mo). Significant heterogeneity was noted in the effect of chemoradiation dependent on resection margin (χ^2 = 4.2, P = 0.04). Adjuvant chemoradiotherapy was estimated to reduce the risk of death by 28% (HR = 0.72, 95%CI: 0.47-1.10) in patients with positive margins, but was estimated to increase the risk by 19% (HR = 1.19, 95%CI: 0.95-1.49) in patients with clear margins.

Liao et al[26]'s recent meta-analysis has been the first to directly compare chemoradiation combined with either 5-FU or gemcitabine with each treatment in isolation. No survival advantage was demonstrated by adding chemoradiation to either adjuvant 5-FU or gemcitabine with all hazard ratios approaching 1. However, the addition of chemoradiation to 5-FU (HR = 2.85, 95%CI: 0.15-61.44) or gemcitabine (HR = 36.49, 95%CI: 0.34-3235.7) dramatically increases toxic events in comparison to the use of the chemotherapeutic agent alone. The authors conclude that on the basis of their results, future trials with chemoradiation are not required citing toxicity, resistance and early tumour dissemination as possible reasons why chemoradiotherapy to the tumour bed may be ineffective in pancreatic cancer.

CURRENT PHASE III TRIALS

It is extremely encouraging to see several phase III trials

currently recruiting for patients to further investigate the role of adjuvant therapy in pancreatic cancer (Table 5). Capecitabine is a fluoropyrimidine which has been shown to exert synergistic antitumour activity when combined with gemcitabine[43]. A meta-analysis of three randomised controlled trials (n = 935)[44] compared gemcitabine with gemcitabine plus capecitabine (Gemcap) in advanced pancreatic cancer. This showed an overall survival benefit with the latter treatment (HR = 0.86, 95%CI: 0.75-0.98, P = 0.02) with no intertrial heterogeneity. The ESPAC group is currently recruiting to trial this therapy in the adjuvant setting with the aim of recruiting nearly 1400 patients by the end of 2014[45].

Folfirinox is another chemotherapeutic regimen that has been subjected to a randomised controlled trial in those with metastatic pancreatic cancer[46]. In comparison to gemcitabine (n = 171), Folfirinox (n = 171) improved both median overall survival from 6.8 to 11.1 mo (HR = 0.57, 95%CI: 0.45-0.73, P < 0.001) and median progression-free survival from 3.3 mo to 6.4 mo (HR = 0.47, 95%CI: 0.37-0.59, P < 0.001). However, Folfirinox resulted in significantly more grades 3 and 4 adverse events than gemcitabine including neutropenia, febrile neutropenia, thrombocytopenia, diarrhoea, and sensory neuropathy. To the contrary of these findings, further data published from this study[47] disclosed that quality of life impairment was significantly reduced in the Folfirinox group compared to gemcitabine indicating its acceptability to patients. Folfirinox is currently subjected to a two armed phase III trial in opposition to gemcitabine in

resected pancreatic cancer[48].

The tyrosine-kinase inhibitor Erlotinib, used in combination with gemcitabine has been shown to improve overall survival and progression-free survival compared to gemcitabine alone in advanced pancreatic cancer in a large phase Ⅲ trial (*n* = 569)[49]. Bao *et al*[50] utilised these two compounds in the adjuvant setting in a phase Ⅱ trial achieving a respectable median disease-free survival of 14.0 mo (95%CI: 8.2-24.5). Furthermore, a single-institution phase Ⅱ trial (*n* = 48) has also shown that erlotinib can be safely utilised alongside capecitabine and chemoradiotherapy[51]. Currently, erlotinib is being trialled both in combination with gemcitabine *vs* gemcitabine alone[52] and in a separate trial this will be followed by a course of either capecitabine or 5-FU chemoradiotherapy[53].

Platinum compounds have been safely used in various pancreatic cancer trials, though particularly encouraging results have been achieved in the neoadjuvant setting. Heinrich *et al*[54] prescribed 28 patients a two month neoadjuvant course of gemcitabine and cisplatin. In addition to evidence of a histological response in over half of patients, surgery following this neoadjuvant regimen was safe, leading to a median survival of 26.5 mo. A separate phase Ⅱ study[55] administered neoadjuvant gemcitabine plus the platinum agent oxaliplatin to 33 patients - 18 with unresectable disease and 15 with borderline resectable disease. Following treatment 13/33 underwent resection with over two-thirds of these patients undergoing an R0 resection. Resection improved median overall survival to 22 mo (95%CI: 14-30) compared to 12 mo (95%CI: 9-15) in those treated non-surgically (*P* = 0.046).

On the basis of these encouraging results, phase Ⅲ trials are now incorporating platinum agents into their chemotherapy regimens. The Hyperthermia European Adjuvant Trial (HEAT) study[56] will compare adjuvant gemcitabine to adjuvant gemcitabine plus capecitabine plus regional hyperthermia treatment - a regime that has previously been utilised with low reported toxicity. It has been shown that heat can increase the cytotoxicity of certain chemotherapeutic agents[57] including gemcitabine[58] in *in vitro* experiment with pancreatic cancer cell lines. One phase Ⅱ study[59] combined gemcitabine with regional heat treatment in the treatment of both metastatic and locally advanced disease. Median survival was 8 mo in the entire study population, but extended to 17.7 mo in those with localised disease. More recently, a retrospective analysis of 23 patients with gemcitabine refractory inoperable disease was published[60] whereby patients received gemcitabine plus cisplatin alongside regional hyperthermia biweekly for four months. Though 21/23 patients suffered from metastatic disease at recruitment, a median overall survival of 12.9 mo was achieved (95%CI: 9.9-15.9).

One of the more recent phase Ⅲ studies to be approved is to trial adjuvant gemcitabine *vs* a combination of gemcitabine and the taxane, nabpaclitaxel[61]. This combination has already been shown to be superior to gemcitabine alone in advanced pancreatic cancer in a large phase Ⅲ trial[62] (*n* = 861), where progression free survival improved from 3.7 to 5.5 mo and overall survival growing from 6.7 to 8.5 mo (HR for disease progression or death 0.69, 95%CI: 0.58-0.82, *P* < 0.001). Though not yet active, the results of this promising large international multicentre trial are already much anticipated.

CONCLUSION

Pancreatic cancer remains a substantial challenge for surgeons and oncologists alike. Surgical resection remains the foundation for any patient with resectable disease. There is now irrefutable evidence that adjuvant chemotherapy improves both overall and disease-free survival and several phase Ⅲ trials are currently in progress aiming to challenge gemcitabine as the gold standard adjuvant drug. The evidence for adjuvant chemoradiotherapy in large phase Ⅲ trials is lacking and can therefore not be recommended as standard therapy. Future adjuvant RCTs will compare approaches using combination therapies to attempt to improve the outlook.

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SIBLINGs and SPARC families: Their emerging roles in pancreatic cancer

Ferda Kaleağasıoğlu, Martin R Berger

Ferda Kaleağasıoğlu, Visiting Scientist at Toxicology and Chemotherapy Unit, German Cancer Research Center, D-69120 Heidelberg, Germany

Ferda Kaleağasıoğlu, Department of Pharmacology, Faculty of Medicine, University of Yeditepe, 34755 Ataşehir/İstanbul, Turkey

Martin R Berger, Toxicology and Chemotherapy Unit, German Cancer Research Center, D-69120 Heidelberg, Germany

Author contributions: Kaleağasıoğlu F and Berger MR contributed equally to this work and wrote the review.

Correspondence to: Martin R Berger, Professor, Toxicology and Chemotherapy Unit, German Cancer Research Center, Im Neuenheimer Feld 581, D-69120 Heidelberg, Germany. m.berger@dkfz-heidelberg.de

Telephone: +49-6221-423310 Fax: +49-6221-423313

Abstract

Pancreatic cancer has a considerably poor prognosis with a 5-year survival probability of less than 5% when all stages are combined. Pancreatic cancer is characterized by its dense stroma, which is involved in the critical interplay with the tumor cells throughout tumor progression and furthermore, creates a barrier restricting efficient penetration of therapeutics. Alterations in a large number of genes are reflected by a limited number of signaling pathways, which are potential targets. Understanding more about the molecular basis of this devastating cancer type regarding tumor microenvironment, distinct subpopulations of cells, epithelial-to-mesenchymal transition and inflammation will lead to the development of various targeted therapies for controlling tumor growth and metastasis. In this complex scenario of pancreatic cancer, especially members of the "small integrin binding ligand N-linked glycoproteins" (SIBLINGs) and "secreted protein acidic and rich in cysteine" (SPARC) families have emerged due to their prominent roles in properties including proliferation, differentiation, apoptosis, adhesion, migration, angiogenesis, wound repair and regulation of extracellular matrix remodeling. SIBLINGs consist of five members, which include osteopontin (OPN), bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein. The SPARC family of modular extracellular proteins is comprised of SPARC/osteonectin (ON) and SPARC-like 1 (hevin); secreted modular calcium binding proteins; testicans and follistatin-like protein. In this review, we especially focus on OPN and ON, elaborating on their special and growing importance in pancreatic cancer diagnosis and prognosis.

Core tip: In this article we review the evidence that the protein families "small integrin binding ligand N-linked glycoproteins" (SIBLINGs) and "secreted protein acidic and rich in cysteine" (SPARC) modulate functions like proliferation, differentiation, apoptosis, adhesion, migration, angiogenesis, wound repair, and regulation of extracellular matrix remodeling. Moreover they play significant roles throughout each stage of pancreatic cancer formation and progression. We discuss, with special reference to osteopontin and osteonectin, how SIBLING and SPARC proteins have attracted growing importance as diagnostic and prognostic tools and discuss their fascinating potential as therapeutic targets.

INTRODUCTION

Pancreatic cancer, the fourth leading cause of cancer-related mortality, has a considerably poor prognosis, as reflected by a median survival of 5-8 mo and a 5-year survival probability of less than 5% when all stages are combined[1,2]. At the time of diagnosis, metastasis has already occurred in most of the patients. Early diagnosis may enable tumor resection; however relapse is still likely due to recurrence at the primary tumor site and distant metastasis. This is affirmed by the observation that the average survival time after neoadjuvant therapy and surgery in patients whose tumor was resectable before neo-adjuvant therapy was similar to that of patients treated with chemotherapy and/or radiotherapy after surgery (23.3 and 20.5 mo, respectively)[2].

Recent advances in understanding the molecular basis of pancreatic cancer development and progression are expected to provide novel therapeutic opportunities. In this regard, the genomic diversity, tumor microenvironment, distinct populations of cancer stem cells (CSCs) resistant to chemo- and radiotherapies, and adaptation of cancer cells to the hypoxic conditions as well as to nutritional deficiency represent potential therapeutic challenges[3].

Pancreatic cancer is characterized by its dense and desmoplastic stroma which is critical for tumor progression and metastasis. Tumor stroma creates a barrier restricting efficient penetration of chemotherapeutics and targeted therapies[4,5]. Furthermore, the stroma and the tumor itself express various proteins, which have proven to be prognostic biomarkers and potential therapeutic targets, as well[6]. These cytokines secreted by pancreatic cancer cells, especially members of the small integrin binding ligand N-linked glycoprotein (SIBLING) and secreted protein acidic and rich in cysteine (SPARC) families are likely to draw a lot of interest for their prominent roles in pancreatic cancer growth.

SIBLINGs are a family of non-collagenous proteins consisting of five members, which include osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein (DMP1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE) (Table 1). The SIBLING family of proteins is principally located in bone and dentin and its members take part in extracellular matrix (ECM) formation and mineralization[7]. OPN is highly expressed in primary pancreatic cancer[8-10]. SIB-LINGs are involved in tumor progression and metastasis by interacting with several integrins and with CD44 to mediate cellular signaling[11,12].

The SPARC family of modular extracellular proteins can phylogenetically be classified into four groups (Table 1), all of which contain the extracellular calcium-binding (EC) and follistatin-like (FS) domains (Table 1): (1) Osteonectin (ON) and SPARC-like 1 (hevin); (2) Secreted modular calcium binding proteins (SMOC) 1 and 2; (3) Testican 1, 2 and 3; and (4) Follistatin-like protein[13-15]. ON is overexpressed in pancreatic cancer stroma[10]. Low or absent stromal expression of ON was correlated with

longer survival rates[6]. ON is involved in remodeling of ECM, morphogenesis, wound repair, and cell proliferation. The other SPARC family member hevin is down-regulated in pancreatic cancer, especially in the late stages and was suggested to function as a tumor suppressor and angiogenesis regulator[10,16-19].

In this review, we elaborate on the role of SIBLING and SPARC family members in pancreatic cancer progression and metastasis with specific emphasis on OPN and ON. We start by describing their signaling pathways, then elaborate on the critical interplay between tumor cells and their microenvironment, and outline the currently available targeted therapies in pancreatic cancer. In the following part, we discuss the impact of SIBLING and SPARC family proteins in pancreatic cancer, including their distribution, interference with signaling pathways, pro- and anti-tumorigenic effects, biomarker roles, and fascinating potential as therapeutic targets.

SIGNALING PATHWAYS IN PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer. The development and progression of PDAC is mediated by the complex crosstalk between the tumor cells and the stromal components, involving various alterations in signaling pathways[20]. The first comprehensive genetic analysis of 24 pancreatic tumors revealed alterations of a large number of genes (63 per tumor). A more recent detailed analysis of 99 tumors identified 26 mutations per patient (range 1-116) as well as substantial heterogeneity with 2016 non-silent mutations and 1628 copy-number variations in 99 patients, affirming the previous findings and furthermore pointing out the potential involvement of axon guidance genes in pancreatic cancer progression[21]. However, dysregulation was limited to 12 core signaling pathways which therefore seem to be more preferable targets as compared to mutated genes[22]. These core pathways, which were genetically altered in most pancreatic cancers, include genes like KRAS and other monomeric GTPases, genes for apoptosis, DNA damage control, regulation of G1/S phase transition, Hedgehog, c-Jun N-terminal kinase, TGF-β, Wnt/Notch, genes for invasion, homophilic cell adhesion and integrin signaling[22].

PANCREATIC CANCER MICROENVIRONMENT

Critical interplay between the tumor cells and the microenvironment

The abundant stroma of pancreatic cancer, which has been termed desmoplasia, is composed of acellular (ECM, soluble proteins like cytokines and growth factors) and cellular [fibroblasts, myofibroblasts, pancreatic stellate cells (PSCs), vascular and immune cells] components[4]. The abundant infiltrating inflammatory cells are particu-

Table 1 Small integrin binding ligand N-linked glycoproteins and secreted protein acidic and rich in cysteine gene families and their members[7,13-15]

Gene family	Member (Aliases)
Small integrin binding ligand N-linked glyco-proteins (SIBLING)	Osteopontin (OPN)/secreted phosphoprotein 1 (SPP1)
	Bone sialoprotein II (BSP)
	Dentin matrix protein 1 (DMP1)
	Dentin sialophosphoprotein (DSPP)
	Matrix extracellular phosphoglycoprotein (MEPE)
Secreted protein acidic and rich in cysteine (SPARC)	SPARC [osteonectin (ON)]/basement-membrane protein 40 (BM-40)
	Hevin (SPARCL1; QR1)
	Secreted modular calcium binding proteins 1 and 2 (SMOC1 and SMOC2)
	Testican 1, 2 and 3/sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1, 2 and 3 (SPOCK1, 2 and 3)
	Follistatin-like protein (FSTL-1)/TGF-beta-simulated clone-36/follistatin-like (TSC-36/Flik)/follistatin-related protein (FRP)/TGF-β inducible protein

larly polymorphonuclear neutrophils, macrophages, and lymphocytes. These immune cells are rich sources of various factors promoting tumor growth and EMT associated with enhanced migration capacity and metastasis[23,24].

The desmoplastic response is regulated throughout cancer initiation and progression stages by dynamic paracrine and autocrine signaling interactions between tumor and host stromal cells[25]. PSCs, the key players in desmoplastic reaction, are star shaped cells residing in periacinar, periductal and perivascular regions of the pancreas[26]. During malignant transformation, PSCs are transformed from a quiescent state into an activated (myofibroblast-like) phenotype which expresses α-smooth muscle actin and ECM proteins and acquires the capacity to proliferate, migrate, contract, phagocytose, and promote tissue repair[27]. Pancreatic cancer cells recruit PSCs to their immediate vicinity, while PSCs inhibit apoptosis and stimulate survival of cancer cells[26,28,29]. Human PSCs (hPSCs) have the capability to intravasate/extravasate, thus accompany cancer cells to distant metastatic sites[30]. The premalignant and malignant cells secrete many paracrine factors like transforming growth factor-beta (TGF-β), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), sonic hedgehog (SHH), epidermal growth factor (EGF), fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs), which activate PSCs, which in turn secrete more ECM proteins, matrix metalloproteases (MMPs), PDGF, FGF, TGF-β, IGF1, small leucine-rich proteoglycans, periostin, collagen I, EGF and heparin sulfate proteoglycans (Figure 1)[25].

Targeted therapies in pancreatic cancer

Currently approved chemotherapeutic drugs for pancreatic cancer are pyrimidine analogs (fluorouracil, capecitabine, gemcitabine), platinum analogs (oxaliplatine), taxanes (paclitaxel and docetaxel), campthotecin analog irinotecan and mitomycine C[31]. The growing need for novel agents was met by understanding the molecular basis of pancreatic cancer which paved the way to modulate aberrant signaling pathways. EMT, a process whereby epithelial cells

acquire mesenchymal characteristics which are associated with increased invasiveness, angiogenesis, resistance to chemotherapy and formation of CSCs, has also emerged as an immensely attractive target[32]. Suppression of tumor promoting inflammation presents another potential target. Inflammation is observed at the early stages of PDAC which progresses *via* an interplay between KRAS mutations and chemokines/cytokines. Upregulated oncogenic and inflammatory pathways intersect in the transcription factors STAT3 and NF-κB, designating them as excellent therapeutic targets[33]. In the light of these findings, recent research has focused on molecular targets like epidermal growth factor receptor (EGFR), VEGF, IGF-1R, mammalian target of rapamycin (mTOR), mitogen activated protein kinase (MEK), cyclooxygenase 2 (COX-2) or proteasome[34,35]. In addition, targeting c-MET or Alk-4/7 up-regulated in CSCs or pathways mediating EMT (Notch, Wnt, Hedhehog, Src and TGF-β) or transcription factors (Zeb1) emerge as viable strategies[36].

The role of EGFR, which is an overexpressed oncogene in 43%-69% of PDAC, is well established in pancreatic cancer progression[37]. EGFR belongs to the receptor tyrosine kinase (RTK) subfamily ErbB/EGFR and regulates downstream signaling pathways including the PI3K/AKT, RAS/MAPK, PLCγ/PKC and STATs pathways. A nuclear EGFR complex has also been reported in pancreatic cancer cell lines, Panc-1 and Colo-357 cells, but it's not yet definitively identified as a true oncogene[38]. Several monoclonal antibodies (mAbs) namely cetuximab, matuzumab, panitumumab, and nimotuzumab which can bind to the extracellular domain of membrane-bound EGFR are under investigation. Smaller molecules like erlotinib and gefitinib can inhibit EGFR tyrosine kinase by competetive blockade of ATP binding. Today, erlotinib is the only targeted therapy which is approved as first line therapy in combination with gemcitabine for locally advanced or metastatic pancreatic cancer[35,39]. Human EGF receptor-2 (HER-2) is a commonly expressed oncogene in pancreatic cancer. Anti-HER-2 therapies include mAbs like transtuzumab and pertuzumab, and tyrosine kinase inhibitors (TKIs) like lapatinib[35].

VEGF is another overexpressed oncogene in 93% of
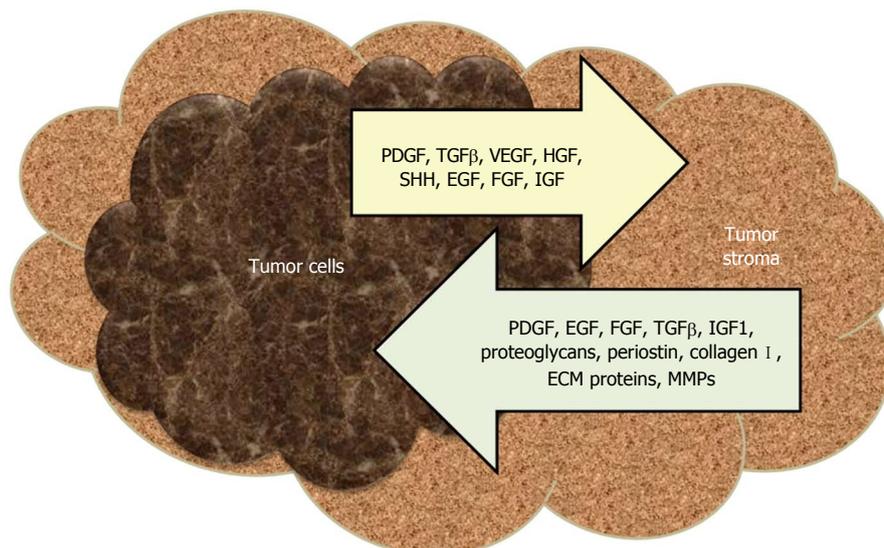
Figure 1 **Critical interplay between the pancreatic cancer cells and the microenvironment.** TGF-β: Transforming growth factor-beta; VEGF: Vascular endothelial growth factor; HGF: Hepatocyte growth factor; SHH: Sonic hedgehog; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor; MMP: Matrix metalloprotease; PDGF: Platelet derived growth factor.

PDAC[37]. Since overexpression of VEGF and its receptors are involved in angiogenic and mitogenic promotion of tumor growth, targeting this pathway with bevacizumab has been evaluated for the treatment of advanced pancreatic cancer combined with other chemotherapeutic regimens[39,40]. An alternative strategy for targeting the VEGF pathway has also been tested using anti-VEGF TKIs sorafenib, axitinib and vatalanib[35].

Other molecularly targeted therapies under investigation are farnesyltransferase inhibitors (tipifarnib), TGF-β signaling inhibitors (TGF-β2 inhibitor AP 12009, dual TGF-β type I / II receptor kinase selective inhibitor LY210976, TβR-I inhibitor LY364947 and selective kinase inhibitor SD-093), IGF-1R kinase inhibitors (NVP-AEW541 and BMS-754807), MMP inhibitors (marimastat and tanomastat), hedgehog signaling inhibitors (cyclopamine, saridegib and vismodegib), mTOR inhibitors (everolimus, temsirolimus, sirolimus), MEK1/2-ATP-uncompetitive inhibitors (selumetinib), COX-2 inhibitors (celecoxib), 26S proteasome inhibitors (bortezomib), NF-κB inhibitors (curcumin), integrin α5β1 inhibitors (volociximab), and a claudin-4 inhibitor (clostridium perfringens enterotoxin).

Pancreatic cancer development and progression is regulated by the interaction between various aforementioned pathways; hence targeting multiple pathways seems to be a novel therapeutic approach to interfere with this cross talk[34,35,41-49].

However, two very recent reviews on targeted therapies indicate a poor outcome in phase III trials in spite of numerous promising results from preclinical studies and phase I / II trials[34,35]. This insurmountable intrinsic and acquired resistance to the investigated therapeutics delineates the critical interplay between tumor cells and tumor microenvironment[5], anticipating the need to identify additional targets as well as novel agents and to specifically

target the tumor stroma[34,50,51].

IMPACT OF SIBLING AND SPARC FAMILIES

Expression of SIBLING and SPARC family members has been associated with pancreatic cancer progression. These cytokines, secreted by pancreatic tumor stromal cells, interfere with various pathways and their expression is associated with survival rates[52-55].

Distribution

SIBLING: OPN is strongly expressed in tumor-associated macrophages especially at the invasive edge of the tumor[8,56,57], in the cytoplasm of tumor cells[53,57,58] and ECM of pancreatic cancer cell lines[59]. BSP is weakly to moderately detectable in islet and ductal cells of normal pancreatic tissues, and in the tubular complexes of PDAC and pancreatic cancer cell lines[60].

SPARC: ON is expressed at high levels by pancreatic acinar and islet cells of normal human tissues[61,62]. In chronic pancreatic inflammation, ON expression in acinar cells is transiently up-regulated but then lost at the final stages, which may favor acinar-to-ductal metaplasia[63]. The majority of pancreatic cancer cells and cell lines are ON negative[54,55,62,64,65]. Lack of ON expression in these cell lines was related to epigenetic silencing by aberrant methylation[62]. The aberrant ON methylation status was not different between sporadic and familial pancreatic cancers[66]. Low-to-absent ON expression levels in some pancreatic cancer cell lines was also associated with overexpression of runt-related transcription factor-2[67] and fibroblast growth factor receptor1-IIIc (FGFR1-IIIc)[68]. ON was overexpressed in stromal fibrocytes and endo-
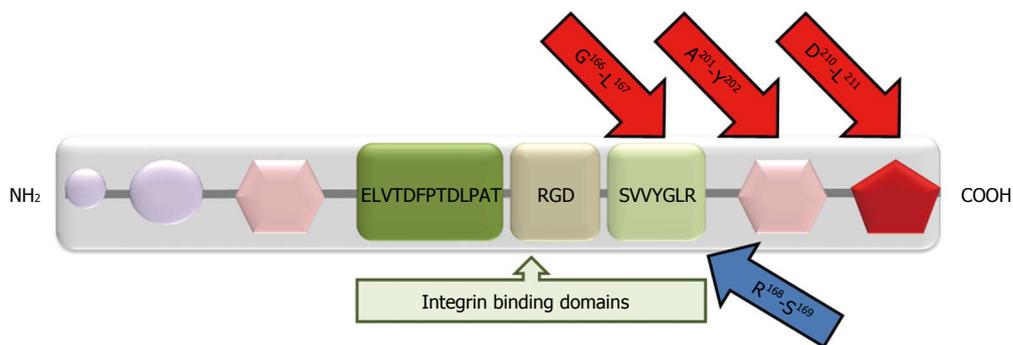
Figure 2 Structural domains of osteopontin. Purple circles: Matrix binding domains; pink hexagons: Calcium binding sites; Red pentagon: Heparin binding site. There are three integrin binding sequences: (1) Arginine-glycine-aspartic acid (RGD); (2) Serine-valine-valine-tyrosine-glutamate-leucine-arginine (SVVYGLR); and (3) ELVTDFPTDLPAT. MMP cleavage sites (G^{166}-L^{167}; A^{201}-Y^{202}; D^{210}-L^{211}) are shown by red arrows. The thrombin cleavage site (R^{168}-S^{169}) is shown by blue arrow.
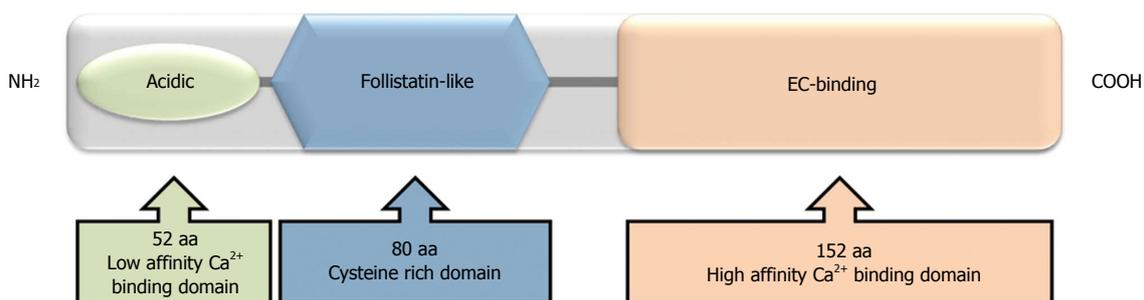

Figure 3 Structural domains of osteonectin. The N-terminal is a highly acidic, calcium binding domain (low affinity). The follistatin-like domain is rich in cysteine residues. The C-terminal is an extracellular calcium-binding domain (high affinity).

thelial cells of benign and malignant tissues, especially adjacent to the neoplastic epithelium but also in the distal stroma[55,61,62,65,69]. Hevin mRNA was expressed specifically within angioendothelium but not in adjacent tumor epithelium and stroma of invasive pancreatic cancer[70].

Interference with signaling pathways in cancer progression

SIBLING and SPARC proteins modulate many functions of healthy tissues, including cell proliferation, differentiation, apoptosis, adhesion, migration, angiogenesis, wound repair, and regulation of ECM remodeling. Mounting evidence suggested their significant functions in various cell-matrix interactions throughout each stage of cancer progression, which include, but are not limited to integrin linked kinase (ILK)/PI3K/Akt, Ras/Raf/MEK/ERK1/2/AP-1 and NF-κB as major signaling pathways[11,13,71].

OPN: OPN is a flexible protein in solution. This capability of OPN allows its binding, *via* Arg-Gly-Asp (RGD) motif-dependent and independent interactions, to different proteins like cell surface receptors, matrix metalloproteinases and ECM proteins[11]. OPN was shown to promote proliferation, invasion, angiogenesis, and metastasis in different types of malignant tumors[71-76]. OPN interacts mainly with various α_v ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_6$) integrin receptors *via* the RGD sequence and with

CD44v6 and v7-containing isoforms *via* the C-terminal fragment with a calcium binding site (Figure 2). Binding of OPN to integrin and CD44 initiates a downstream signaling cascade through the PI3K/Akt signaling pathway leading to NF-κB mediated cell proliferation and survival[71,73]. An OPN/integrin complex, through the Ras/Raf/MEK/ERK pathway, activates AP-1 dependent gene expression, hence plasmin and MMP-9 mediated ECM degradation and tumor invasion[71]. VEGF-induced OPN and integrin expression supports neovascularization processes by promoting endothelial cell migration and vascular lumen formation, activating monocytes to release pro-angiogenic cytokines and preventing endothelial cell apoptosis[73].

ON: ON has three structural domains (Figure 3), each of which initiates differential processes in cancer progression. The N-terminal, highly acidic low affinity-calcium binding domain inhibits cell migration and chemotaxis, decreases fibronectin and thrombospondin-1 but increases plasminogen activator inhibitor-1 (PAI-1). The cysteine rich follistatin-like domain promotes de-adhesion, angiogenesis and proliferation and the high affinity-EC-binding domain inhibits migration, proliferation and adhesion, induces MMPs and regulates cell-matrix interactions[77,78]. Tumors overexpressing the N-terminal domain of ON were used as model to show that this domain has chemosensitizing properties. In fact, the N-terminal domain of ON caused a significantly greater reduction in
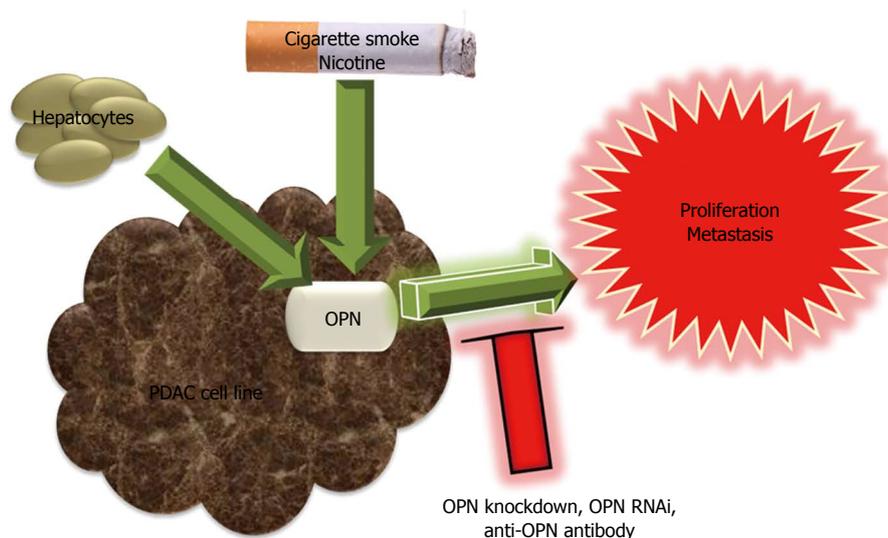
Figure 4 Protumorigenic role of osteopontin in pancreatic cancer development and progression. Osteopontin (OPN) expression in pancreatic cancer cell lines is associated with increased *in vitro* proliferation and enhanced growth and metastasis *in vivo*, which are reversed by OPN knockdown, OPN RNAi and anti-OPN antibody. Exposure to cigarette smoke (including nicotine) and hepatocytes induce OPN expression.

cell viability than ON itself and this effect was related to enhancing the apoptotic cascade *via* activation of caspase 8[79].

ON has a divergent effect in different cancer types. ON may be linked with a highly aggressive phenotype in some tumors, but it may function as a tumor suppressor in others[80]. This modulator effect, either positive or negative, on cell growth and migration was suggested to depend on the amount secreted by the tumor[81]. The microenvironment also determines whether ON will act as a de-adhesive or adhesive protein[82]. ON influences integrin signaling by reducing surface localization of integrin subunits and by directly interacting with ILK and it induces ILK/FAK/Akt activation to promote EMT, anti-apoptosis and cell migration[71,77]. Macrophage-derived ON is also involved in integrin-mediated metastasis[83]. ON can bind directly to collagens Ⅰ-Ⅷ and growth factors (VEGF-A, PDGF-AA and PDGF-BB) or modulate cell surface receptors of basic fibroblast growth factor and TGF-β[13,77,78,84,85]

Prominent roles in pancreatic cancer progression: experimental evidence

OPN: OPN has a pro-tumorigenic role and favors the metastatic growth of pancreatic cancer (Figure 4). OPN mRNA expression in human PDAC cell lines was significantly related to their growth in the liver of nude rats. Similarly, OPN knockdown was associated with reduced proliferation in rat pancreatic adenocarcinoma (AsML) cells[86]. OPN expression in AsML cells following explantation from the liver decreased gradually in time when grown in vitro for up to five weeks. However, co-culture of AsML cells and of human Suit2-007 PDAC cells with hepatocytes stimulated OPN expression. This two compartmental metastasis model clearly demonstrated the cross talk between PDAC cells and hepatocytes[86]. Comparison of OPN expression in two human pancreatic

cell lines showed that OPN was eleven-fold up-regulated in cells of the highly liver metastatic cell line HPC-3H4, as compared to parental HPC-3 cells. In the same study, OPN RNAi and anti-OPN antibody treatment inhibited liver metastasis of pancreatic cancer cell line[87]. OPN was likely to play a role in the initial growth of PDAC cells in the liver while MMP-1 and EGF-1 were required for the maintenance of growth[88].

OPN was demonstrated to be a downstream mediator of nicotine in pancreatic cancer. Smoking and experimental exposure to cigarette smoke or nicotine stimulated OPN mRNA and protein expression in PDAC[89]. Nicotine exposure selectively induced splice variant OPNc and alpha7-nicotine acetylcholine receptor (α7-nAChR) expression[90]. Nicotine-induced OPN mRNA expression in pancreatic cancer cell lines was inhibited by a nicotinic acetylcholine receptor antagonist, a tyrosine kinase inhibitor or an ERK1/2 activation inhibitor, which implied that OPN was expressed by a nAChR-ERK1/2-dependent pathway[89]. OPN siRNA or antibody treatment inhibited nicotine enhanced expression of MMP-9 and VEGF[91].

ON: ON is a multifaceted protein with controversial functions of its structural domains. ON exhibited differential effects in pancreatic cancer models and displayed antitumorigenic as well as tumorigenic potential (Figure 5). The experimental models used providing differential environmental conditions as well as cancer cell line specific properties seem to contribute to its complex behavior.

The antitumorigenic potential of ON in pancreatic cancer was demonstrated by *in vivo* and *in vitro* studies. An inverse relation exists between ON expression and growth of pancreatic cell lines in the liver of nude rats[86]. ON expressing human Suit2-007 and rat AsML cells were investigated *in vitro* to elucidate the antiproliferative role of ON. Knockdown of ON mRNA by an antisense
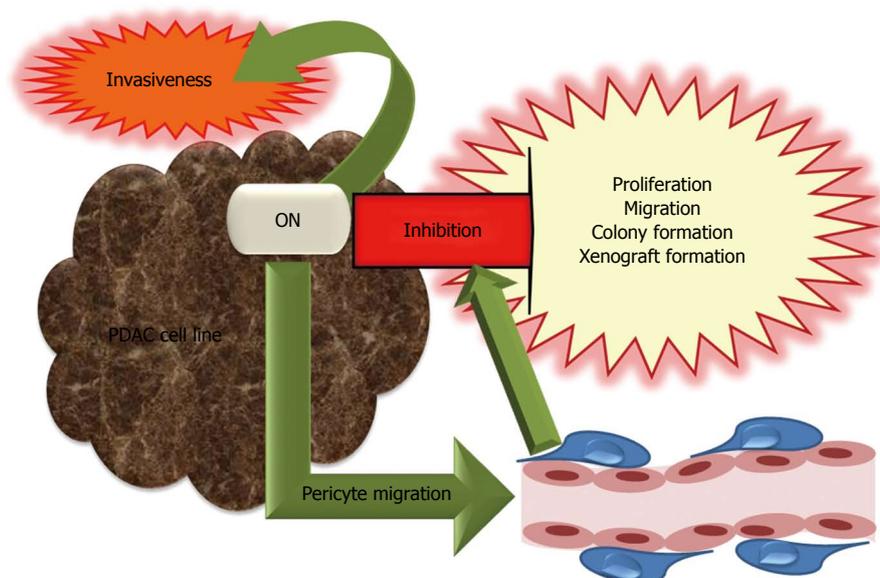
Figure 5 Pro- and anti-tumorigenic roles of osteonectin in pancreatic cancer development and progression. Osteonectin (ON) is a multifaceted protein with controversial functions of its structural domains. ON is anti-tumorigenic and inhibits proliferation, migration, colony and xenograft formation as well as invasiveness. Facilitation of pericyte migration by ON contributes to inhibition of tumor spread. However, ON is also pro-tumorigenic and induces invasiveness.

oligonucleotide (ASO) in Suit2-007 cells was associated with increased proliferation as compared to a nonsense control. Accordingly, human recombinant ON decreased proliferation of AsML and Suit2-007 cells in a time and concentration dependent manner[86]. In other studies, exogenous ON also caused dose-dependent inhibition of proliferation in PDAC cell lines[65,68] and this effect was independent of endogenous ON expression[68]. ON-induced growth arrest was associated with increased p21 expression, which induces a G1 cell cycle block[65]. Furthermore, inhibition of endogenous ON by small hairpin RNA resulted in enhanced cell proliferation, migration, colony formation and xenograft formation[68]. The mechanism by which ON might promote apoptosis was investigated in the chemotherapy resistant pancreatic cell line MiaPaca/CPT, which showed that caspase 8-Bcl2 interaction was abolished following ON exposure and restored by treatment with ON antibodies[79]. The anti-tumorigenic effect of ON was confirmed by *in vivo* studies, *e.g.*, ON expression reduced tumor invasiveness as shown in an orthotopic murine model of pancreatic adenocarcinoma. ON-null mice had larger and more invasive tumors with reduced MMP-9 expression, ECM deposition, and microvessel density as compared to the wild type[92]. However, reduced vessel density in ON-null mice was not accompanied with altered levels of VEGF and TGF-β1. Tumor spread in ON-null mice was explained by reduced ECM deposition (with less mature and/or collagen fibrils), decreased pericyte recruitment, disrupted vascular basement membrane and reduced apoptosis[93,94]. The interaction of ON with pericytes was investigated in an in vitro model utilizing primary pericytes isolated from ON positive or null mice pancreatic tissues or 10T1/2 cells which can differentiate into mesenchymal lineages. It was proposed that ON could facilitate pericyte migration

by preventing interaction of endoglin, a TGF-β1 accessory receptor with αv integrins[95]. Accordingly, aberrant TGF-β1 activation in ON-null mice led to significant tumor progression[96].

However, ON also displayed tumorigenic potential. For example, ON treatment increased the invasive capacity of pancreatic adenocarcinoma cell lines *in vitro*. Specifically, the invasive capacity of the ON expressing human metastatic cell line Colo-357 was increased 14 fold in response to ON exposure. Down-regulation of ON expression by ASO reduced the in vitro invasiveness of Panc-1 cells[65]. Exogenous ON-induced invasiveness was observed in monoculture of Panc-1 cells as well as their co-culture with hPSCs[55]. Direct MMP-2 induction by exogenous ON was suggested to account for promotion of tumor invasion in PDAC[65].

Biomarker role for pancreatic cancer

A compendium of potential biomarkers for pancreatic cancer was developed, which includes 2516 genes. It was reported that 441 genes were overexpressed (defined as an at least two-fold increase or if shown by multiple methods) both at mRNA and protein levels. OPN was listed by more than four studies and therefore was among the best potential biomarker candidates for pancreatic cancer, deserving focused validation[10] in line with its proven value as a clinical tumor progression marker for several forms of cancer[97]. In the same compendium, among 266 genes overexpressed in cancer cells as well as in stroma, only 5 were expressed only in stroma. ON is among the small number of molecules which are overexpressed only in stroma[10].

OPN: The diagnostic and prognostic impact of OPN expression was highlighted by various studies. Using

quantitative reverse transcription-polymerase chain reaction (qRT-PCR), OPN expression was shown to increase by 13.1 fold in the parenchyma adjacent to infiltrating cancer relative to normal pancreatic parenchyma, whereas this increase remained at 5.3 fold in the parenchyma adjacent to chronic pancreatitis. Therefore OPN was identified as a helpful predictor of pancreatic cancer lesions[98]. A recent study on surgical specimens from patients with PDAC demonstrated stronger immunostaining for OPN expression with advanced grades[99].

Serum OPN levels were also significantly elevated in pancreatic cancer patients as compared to healthy control subjects[8,100-103]. A pilot study evaluated 12 blood biomarker candidates for detection of pancreatic cancer and demonstrated that macrophage migration inhibition factor and OPN blood tests (with 100% and 95% sensitivity, respectively) were almost perfect to distinguish pancreatic cancer cases from healthy individuals[102]. Specifically, the OPN splice variants OPNb and OPNc were increased in pancreatic cancer when compared to non-cancer controls, as assessed by RT-PCR blood test[9]. OPNc, which supported anchorage independence, was suggested to be the most potent OPN isoform for the pro-metastatic behavior, hence a candidate marker for invasive PDAC[104].

Serum OPN levels in advanced stages III and IV were higher than in early stages I and II, indicating that OPN may be a useful diagnostic marker to distinguish resectable cases and to predict survival rates[101]. Cytoplasmic OPN expression was not correlated with average tumor size, tumor stage, and nodal status[53,58]. The improved survival when OPN was expressed in the cytoplasm (17.1 mo vs 11.6 mo) was linked to a relatively small size (< 2 cm) of OPN positive tumors. Therefore, it was suggested that OPN expression might be lost as tumors grow and turn into an aggressive phenotype[53].

ON: In experimental models based on pancreatic cell lines, both, an antitumorigenic and tumorigenic potential of ON was demonstrated, and studies in humans showed that stromal expression of ON is particularly important for the prognosis of these patients. The ON expression pattern was investigated by immunohistochemical analysis in 299 primary PDAC resection specimens from patients who underwent pancreatico-duodenectomy. ON expression by stromal fibroblasts was found to be a strong marker of poor prognosis. Median survival in ON (+) patients was decreased by 50% (15 mo) as compared to ON (-) patients (30 mo)[54]. Immunohistochemical analysis in 58 biopsy specimens from patients with locally advanced pancreatic cancer showed an inverse correlation between stromal ON expression and overall survival (OS). Therefore ON expression in non-resectable tumor stroma was strongly indicative of a poor prognosis[55]. In another trial, stromal ON expression on 5-year survival rate was evaluated. It was shown that the 5-year survival rate in patients with a low ON mRNA level was better (23%) as compared to those with high ON mRNA level. However, no significant correlation was found between stromal ON mRNA overexpression and depth of tumor invasion, lymph node metastasis, stage, histopathological tumor grade, lymphatic invasion, vascular invasion or surgical margin, (0%)[105]. A recent prospective randomized phase III study including 160 patients treated with curatively intended resection and receiving adjuvant treatment with gemcitabine, demonstrated that disease-free and overall survival decreased with strong ON expression. In contrast to previous reports, this finding was not only related to the peritumoral stroma (strong vs not strong DFS 9.0 mo vs 12.6 mo and OS 19.8 mo vs 26.6 mo) but also to the cytoplasmic ON expression of adenocarcinoma cells (positive vs negative DFS 7.4 mo vs 12.1 mo and OS 14.1 mo vs 25.6 mo)[106]. ON expression was shown to be a predictive marker independent of CA19-9 levels[107].

Altered methylation patterns of ON gene transcriptional regulation region were suggested for use as a tumorigenesis marker for early detection of pancreatic cancer. In a small scale study comprising 40 cases of pancreatic cancer and the adjacent normal tissues, 6 chronic pancreatitis tissues, and 6 acute pancreatic tissues, all were analyzed by bisulfite-specific PCR based sequencing. As a result, aberrant hyper-methylation of CpG region 1 and, especially, CpG region 2 might be an early step in pancreatic cancer development and progression and differentiate malignant tissues from healthy and chronic pancreatitis tissues[108].

Hevin: Hevin mRNA and protein levels were found to be high in bulk PDAC and pancreatic neoplasms[10,18,19]. However, its expression is related to the vascular content of a given lesion. Higher percentages of Hevin positive vessels were detected in chronic pancreatitis (32%) and benign and borderline pancreatic tumors (40%) as compared to PDAC (15%). Down-regulation of Hevin is observed in the late stages of pancreatic cancer progression[18].

Therapeutic targeting in pancreatic cancer

SIBLING and SPARC family members are matricellular proteins, which modulate many critical cellular processes like proliferation, migration, and angiogenesis. For this reason, they represent potential therapeutic targets for either stromal depletion or blockade of signaling pathways involved in pancreatic cancer progression.

Inhibition of metastasis: Since down-regulation of OPN reduces pancreatic cancer cell invasion, OPN has been suggested as a therapeutic target to inhibit metastasis[109].

Stromal depletion: ON and gp60 are functionally and immunologically related albumin binding proteins, which mediate trans-endothelial transportation of albumin via activation of caveolin-1 and formation of caveoli[110-112]. This function renders ON a promising target for stromal depletion of pancreatic tumors. Promising results were obtained by a new nanoparticle albumin-bound formula-

tion of paclitaxel, namely *nab*-paclitaxel. *Nab*-paclitaxel is transported to malignant tissues by albumin, where it is sequestered by ON. Approximately 10-fold endothelial binding and 4-fold transport across the endothelial cell monolayer was achieved by *nab*-paclitaxel as compared to conventional paclitaxel[111]. The combination of gemcitabine plus *nab*-paclitaxel was evaluated in 36 patients with previously untreated advanced pancreatic cancer. Median OS increased significantly in the group with high ON expression when compared to the low-ON group (17.8 mo *vs* 8.1 mo). Improved survival was correlated with ON overexpression in the stroma but not in the tumor. In the same study, the intratumoral gemcitabine concentration was increased nearly 3-fold in mice harboring PANC265 xenografts, derived from 11 chemotherapy-naive patients, when *nab*-paclitaxel was added to gemcitabine treatment. *Nab*-paclitaxel alone and in combination with gemcitabine caused depletion of desmoplastic stroma with resultant vasodilation, which together helped to achieve an increased gemcitabine penetration into the tumor, hence a better response[107].

Improvement of oncolytic activity: Tumor-associated ON positive stromal cells were proposed as potential targets to improve the oncolytic efficacy of conditionally replicative adenoviruses (CRAd). ON positive transformed human microendothelial (HMEC-1) cells enhanced the oncolytic activity of CRAd, Ad(I)-F512-TK, on the ON-negative pancreatic cancer cell line MIA PaCa-2 *in vivo*. Similarly, the *in vitro* oncolytic activity of CRAd increased when MIA PaCa-2 cells were incubated in HMEC-1 and fibroblast (WI-38) conditioned media[113].

Modulation of pericyte migration: Modulation of pericyte recruitment may present a potential therapeutic strategy for increasing the effectiveness of an anti-angiogenic tumor therapy[95]. For example, PDGF-BB overexpression in subcutaneous or orthotopic pancreatic tumors in mice was accompanied with high pericyte content and decreased tumor growth. Therefore, increasing the pericyte content of the tumor microenvironment or targeting PDGFR signaling in tumor associated PDGFR+ pericytes with kinase inhibitors yielded promising results in experimental pancreatic cancer models[114,115]. Likewise, ON is involved in pericyte modulation. ON was shown to promote pericyte migration by inhibiting endoglin-dependent TGF-β1 activity in pancreatic cancer[95]. In parallel with this finding, losartan, which diminishes TGF-β1 activation, was able to slow pancreatic tumor progression[96]. Further studies will elaborate how pericyte modulation can be improved *via* ON targeting[95].

CONCLUSION

Experimental and clinical evidence denote the emerging role of the SIBLING and SPARC family of proteins in pancreatic cancer formation, progression, and metastasis. The differential expression of these proteins in healthy and tumor tissues and correlation of their serum or tumor expression levels with survival rates has shown that they can be useful diagnostic and prognostic biomarkers. ON seems to be a promising target for stromal depletion and anti-angiogenic therapy of pancreatic tumors. Future studies and development of novel agents targeting SIBLING and SPARC family of proteins may help to improve therapeutic response in pancreatic cancer.

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S-1 in the treatment of pancreatic cancer

Kentaro Sudo, Kazuyoshi Nakamura, Taketo Yamaguchi

Kentaro Sudo, Kazuyoshi Nakamura, Taketo Yamaguchi, Department of Gastroenterology, Chiba Cancer Center, Chiba 260-8717, Japan
Author contributions: Sudo K performed the literature search, performed data analysis, and wrote the manuscript; Nakamura K and Yamaguchi T performed data analysis and edited the manuscript.
Correspondence to: Kentaro Sudo, MD, PhD, Department of Gastroenterology, Chiba Cancer Center, 666-2 Nitona-cho, Chuo-ku, Chiba 260-8717, Japan. kentarosudo9@yahoo.co.jp
Telephone: +81-43-2645431 Fax: +81-43-2628680
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Core tip: This review article focuses on clinical trials of S-1-based chemotherapy for advanced pancreatic cancer. Recently, S-1 has been demonstrated to be non-inferior to gemcitabine in overall survival for metastatic or locally advanced pancreatic cancer in a large-scale phase Ⅲ study (GEST study). Furthermore, S-1 has been shown to be superior to adjuvant chemotherapy with gemcitabine in overall survival in patients with resected pancreatic cancer in another phase Ⅲ study (JASPAC-01 study). In addition to gemcitabine, S-1 is now considered one of the key drugs in Japan.

Abstract

S-1 is an oral 5-fluorouracil (5-FU) prodrug, which is designed to improve the antitumor activity of 5-FU by inhibiting dihydropyrimidine dehydrogenase, the key enzyme of 5-FU catabolism. Recently, two important studies on the clinical use of S-1 for pancreatic cancer have been reported from Japan. In the first study (GEST study), S-1 demonstrated non-inferiority to gemcitabine (GEM) in overall survival (OS) for metastatic or locally advanced pancreatic cancer, but combination chemotherapy with GEM and S-1 did not show superiority to GEM in OS. In the second study (JASPAC-01 study), S-1 showed superiority to adjuvant chemotherapy with GEM in OS in patients with resected pancreatic cancer. In addition to GEM, S-1 is now regarded as the key drug in the management of pancreatic cancer in Japan. To date, many studies have investigated the effectiveness of S-1 in various settings, such as first-line chemotherapy for metastatic or locally advanced pancreatic cancer, second-line chemotherapy after GEM failure, and chemoradiotherapy for locally advanced disease. In this review, we focus on recent clinical trials of S-1-based chemotherapy for advanced pancreatic cancer.

INTRODUCTION

Pancreatic cancer is one of the most fatal malignancies worldwide. Gemcitabine (GEM) is accepted as the standard treatment in the management of pancreatic cancer based on a randomized controlled study reported by Burris *et al*[1] in 1997. In an effort to improve therapeutic efficacy, many clinical trials have been conducted. However, the prognosis of patients with pancreatic cancer still remains poor, with a reported 5-year survival rate of less than 10%[2]. Development of more effective therapies is urgently needed.

S-1 is an oral 5-fluorouracil (5-FU) prodrug that consists of tegafur (a prodrug of 5-FU), gimeracil [a potent dihydropyrimidine dehydrogenase (DPD) inhibitor], and oteracil (an inhibitor of phosphorylation of 5-FU in

Table 1 Pivotal phase III studies of first-line chemothrapy for advanced pancreatic cancer

Ref.	Treatment	n	ORR	PFS/TTP (mo)	MST (mo)
Burris *et al*[1]	GEM	63	5.4%	9 wk[b]	5.65[b]
	5-FU	63	0%	4 wk	4.41
Moore *et al*[13]	GEM	284	8%	3.55	5.91
	GEM + erlotinib	285	8.6%	3.75[d]	6.24[e]
Conroy *et al*[14]	GEM	171	9.4%	3.3	6.80
	FOLFIRINOX	171	31.6%[d]	6.4[d]	11.1[d]
Von Hoff *et al*[15]	GEM	430	7%	3.7	6.70
	GEM + nab-paclitaxel	431	23%[d]	5.5[d]	8.50[d]

[b]$P < 0.01$ *vs* 5-FU; [d]$P < 0.01$ *vs* GEM; [e]$P < 0.05$ *vs* GEM. PFS: Progression-free survival; TTP: Time to progression; MST: Median survival time; GEM: Gemcitabine; ORR: Objective response rate; 5-FU: 5-fluorouracil; FOLFIRINOX: Oxaliplatin, irinotecan, fluorouracil and leucovorin.

Table 2 Phase II studies of Gemcitabine and S-1 therapy for advanced pancreatic cancer

Ref.	n	Disease extent	ORR	PFS/TTP (mo)	MST (mo)
Nakamura *et al*[19]	33	Metastatic	48%	5.4	12.5
Ueno *et al*[20]	54	Metastatic	44.4%	5.9	10.1
Oh *et al*[21]	38	Metastatic or LA	32%	5.4	8.4
Lee *et al*[22]	32	Metastatic or LA	44%	4.92	7.89
Kim *et al*[23]	22	Metastatic or LA	27.3%	4.6	8.5

PFS: Progression-free survival; TTP: Time to progression; MST: Median survival time; LA: Locally advanced; ORR: Objective response rate.

the gastrointestinal tract) in a 1:0.4:1 molar concentration ratio[3]. After oral ingestion, tegafur is transformed into 5-FU in the liver. Gimeracil inhibits the degradation of 5-FU by inhibiting DPD, the key enzyme of 5-FU catabolism. In a preclinical study, the DPD inhibitory effect of gimeracil has been shown to be approximately 180-fold more potent than that of uracil, a DPD inhibitor combined in UFT[4]. Therefore, sufficient concentrations of 5-FU in serum and tumor tissues can be maintained. Oteracil inhibits phosphorylation of 5-FU in the gastrointestinal tract, and it is expected to reduce 5-FU-induced gastrointestinal toxicity, which may be observed in parallel with potentiated antitumor activity[5]. Clinically, S-1 is accepted as a convenient alternative to 5-FU continuous infusion in Japan because phase III studies have shown that S-1-based regimens are non-inferior to 5-FU infusion regimens[6-8]. When compared to 5-FU continuous infusion, oral administration of S-1 can avoid the risk of complications associated with central venous catheter placement. Furthermore, for advanced gastric cancer, an S-1-based regimen (S-1 plus cisplatin) is now accepted as the standard first-line chemotherapy in Japan based on the result of a randomized controlled trial[9].

S-1 has been approved for the treatment of pancreatic cancer since 2006 in Japan, and various clinical trials have been conducted. Recently, two important studies on the clinical use of S-1 for pancreatic cancer have been reported. The first study evaluated the effectiveness of S-1 in first-line chemotherapy for metastatic or locally advanced pancreatic cancer[10]. The second study investigated the use of S-1 in adjuvant chemotherapy for resected pancreatic cancer[11,12]. In this review, we focus on recent clinical trials of S-1-based chemotherapy for advanced pancreatic cancer.

FIRST-LINE CHEMOTHERAPY WITH S-1 FOR METASTATIC OR LOCALLY ADVANCED PANCREATIC CANCER

GEM has been the mainstay in the treatment of meta-static or locally advanced pancreatic cancer. However, the reported median survival of first-line GEM therapy is only approximately 6 to 7 mo[1,13-15]. GEM plus erlotinib is the first combination chemotherapy that has demonstrated significantly improved OS compared to GEM alone in this patient population[13]. Additionally, the FOLFIRINOX regimen (oxaliplatin, irinotecan, fluorouracil, and leucovorin) and GEM plus nanoparticle albumin-bound paclitaxel (nab-paclitaxel) regimen have now emerged as aggressive treatment options in patients with metastatic pancreatic cancer (Table 1)[14,15].

The first phase II study of S-1 for pancreatic cancer was reported by Ueno *et al*[16] in 2005. In this study, 19 patients with metastatic pancreatic cancer received S-1 twice daily at a dose of 80, 100, or 120 mg/d according to body surface areas for 28 consecutive days followed by a 14-d rest. Four patients (21.1%) achieved partial response, and the median survival was 5.6 mo. Subsequently, Okusaka *et al*[17] reported a phase II study for metastatic pancreatic cancer, in which a single agent of S-1 showed promising efficacy with a response rate of 37.5% and median survival of 9.2 mo. The major adverse events were gastrointestinal toxicity, such as anorexia, nausea, or diarrhea.

Combination chemotherapy of GEM and S-1 (GS) has also been investigated on the basis of the preclinical findings that GEM and 5-FU have a synergistic cytotoxic effect against pancreatic cancer cells[18]. Phase II studies of GS therapy have also shown favorable efficacy with a response rate of 27.3%-48% and median survival of 7.89-12.5 mo (Table 2)[19-23].

Based on these results, a large-scale phase III study (GEST study) was conducted in patients with metastatic or locally advanced pancreatic cancer in Japan and Taiwan[10]. Between 2007 and 2009, 834 patients were randomly assigned to GEM alone, GS, or S-1 alone. The primary endpoints were superiority of GS therapy to GEM alone in OS and non-inferiority of S-1 to GEM in OS. In the GEST study, the median survival was 8.8 mo for GEM, 9.7 mo for S-1, and 10.1 mo for GS. The non-inferiority of S-1 to GEM was confirmed (HR = 0.96; 97.5%CI: 0.78-0.18; $P < 0.001$). Meanwhile, GS therapy did not demonstrate the superiority to GEM in OS (HR = 0.88; 97.5%CI: 0.71-1.08; $P = 0.15$). Based on the re-

Table 3 Randomized studies of gemcitabine and S-1 therapy *vs* gemcitabine for metastatic or locally advanced pancreatic cancer					
Study	**Treatment**	**n**	**ORR**	**PFS (mo)**	**MST (mo)**
GEST[10]	GS	275	29%[b]	5.7[b]	10.1
(PⅢ)	S-1	280	21%[a]	3.8[c]	9.7[d]
	GEM	277	13%	4.1	8.8
GEMSAP[24]	GS	53	18.9%	5.4[a]	13.5
(rPⅡ)	GEM	53	9.4%	3.6	8.8
JACCRO PC-01[25]	GS	53	28.3%[b]	6.15[b]	13.7[a]
(rPⅡ)	GEM	59	6.8%	3.78	8.0
Sudo *et al*[26]	GS	51	21.6%[a]	5.3[a]	8.6
	GEM	50	6.0%	3.8	8.6

[a]P < 0.05 *vs* GEM; [b]P < 0.01 *vs* GEM; [c]P < 0.05 non-inferiority to GEM; [d]P < 0.01 non-inferiority to GEM. PFS: Progression-free survival; MST: Median survival time; GEM: Gemcitabine; GS: Gemcitabine and S-1; ORR: Objective response rate.

Table 4 Phase Ⅱ studies of S-1 for gemcitabine refractory pancreatic cancer				
Ref.	**n**	**ORR**	**PFS (mo)**	**MST (mo)**
Morizane *et al*[34]	40	15.0%	2.0	4.5
Sudo *et al*[35]	21	9.5%	4.1	6.3

PFS: Progression-free survival; MST: Median survival time; ORR: Objective response rate.

sults of the GEST study, S-1 is accepted as an option in the treatment of metastatic or locally advanced pancreatic cancer in Japan.

However, there are no definitive criteria for the use of S-1 instead of GEM in first-line chemotherapy for metastatic or locally advanced pancreatic cancer. In this regard, there are several important observations in the GEST study results. The first is the difference in toxicity profile between GEM and S-1. Gastrointestinal toxicity, such as diarrhea, was more frequent in the S-1 arm, while hematologic toxicity was more frequent in the GEM arm. The second is the difference in objective tumor response rate. S-1 showed favorable objective response rate (ORR) compared to GEM alone in the GEST study (21% *vs* 13.3%, $P = 0.02$)[10].

GS therapy significantly improved progression-free survival (PFS) (HR = 0.66; 97.5%CI: 0.54-0.81, $P < 0.001$; median, 5.7 mo *vs* 4.1 mo) and ORR (29.3% *vs* 13.3%; $P < 0.001$) compared to GEM alone in the GEST study[10]. Other small-randomized studies reported from Japan also support the superiority of GS therapy to GEM alone with respect to PFS (Table 3)[24-26]. In contrast, the superiority of GS therapy in OS was not demonstrated in the GEST study. The authors of the GEST study explained the cause for the discrepancy as a consequence of second-line chemotherapy with S-1 in the GEM group. Indeed, approximately 50% of patients in the GEM group received second-line chemotherapy with S-1-based regimens[10]. Considering the results of the GEST study, which is the only phase Ⅲ study with the primary endpoint of OS, GS therapy (as well as other GEM and fluoropyrimidine combinations) is not accepted as the standard chemotherapy for metastatic or locally advanced pancreatic cancer in Japan[27-29]. In contrast, some investigators consider that GS therapy may be beneficial in a selected patient population based on the following two studies. A meta-analysis of GS therapy *vs* GEM alone, including the GEST study and two randomized phase Ⅱ studies (GEMSAP and JACCRO), has suggested that GS therapy is associated with better OS (HR = 0.79)[30]. In addition, a pooled analysis of the above three studies has shown that GS therapy significantly im-

proves OS, especially in locally advanced pancreatic cancer (HR = 0.708; 95%CI: 0.527-0.951)[31].

SECOND-LINE THERAPY AFTER GEMCITABINE FAILURE

It is important to establish effective second-line therapies for tumors refractory to GEM. The results of a randomized controlled study reported by Pelzer *et al*[32] have provided the first evidence for the benefit of second-line chemotherapy compared to best supportive care (BSC) alone in patients with GEM refractory pancreatic cancer. Although the study was terminated because of insufficient accrual, oxaliplatin, folinic acid, and 5-FU (OFF) significantly improved second-line survival compared to BSC alone (median, 4.82 mo *vs* 2.3 mo; $P = 0.008$)[32]. Moreover, the results of the CONKO-003 study have demonstrated a significant improvement in OS with the addition of oxaliplatin to 5-FU plus folinic acid[33]. In clinical practice, fluoropyrimidine based therapy is commonly used for patients previously treated with GEM.

Phase Ⅱ studies of S-1 in patients with GEM-resistant pancreatic cancer have demonstrated moderate activity (ORR = 9.5%-15%; Disease control rate, 52%-58%; median survival, 4.5-6.3 mo) with acceptable toxicity (Table 4)[34,35]. Although there has been no confirmed evidence based on phase Ⅲ studies, S-1 would be a feasible treatment option in this patient population.

S-1-based combination regimens have also been investigated (Table 5). Mizuno *et al*[36] reported a randomized phase Ⅱ trial of S-1 *vs* S-1 plus irinotecan (IRIS) in which 127 patients were randomly assigned to IRIS or S-1 alone. The primary endpoint was PFS. IRIS did not improve PFS (HR = 0.767; 95%CI: 0.527-1.114; $P = 0.1750$) or OS (HR = 0.749; 95%CI: 0.512-1.093; $P = 0.1338$) compared to S-1 alone. Okusaka *et al*[37] reported a randomized phase Ⅱ study of S-1 plus oxaliplatin (SOX) *vs* S-1 in patients with GEM refractory pancreatic cancer. The primary endpoint was PFS, and 264 patients were randomly assigned to SOX or S-1 alone. However, SOX did not improve PFS (HR = 0.838; 95%CI: 0.649-1.082; $P = 0.1795$) or OS (HR = 1.031; 95%CI: 0.791-1.344; $P = 0.8235$) compared to S-1 alone.

More recently, the results of a randomized phase Ⅱ study of S-1 plus leucovorin (SL) *vs* S-1 alone in patients with GEM refractory advanced pancreatic cancer have been reported[38]. In this study, SL significantly improved PFS, which was the primary endpoint of this study, compared to S-1 alone (HR = 0.56; 95%CI: 0.37-0.85; $P =$

Table 5 Randomized phase Ⅱ studies of S-1 based chemotherapy after gemcitabine failure

Ref.	Treatment group	n	ORR	PFS (mo)	MST (mo)
Mizuno *et al*[36]	S-1 + irinotecan	60	18.3%	107 d	208 d
	S-1	67	6.0%[a]	58 d	176 d
Okusaka *et al*[37]	S-1 + oxaliplatin	134	20.9%	3.0	7.5
	S-1	130	11.5%[a]	2.8	7.0
Okusaka *et al*[38]	S-1 + leucovorin	69	27.5%	3.8	6.3
	S-1	71	19.7%	2.7[b]	6.1

[a]$P < 0.05$ *vs* S-1; [b]$P < 0.01$ *vs* S-1. PFS: Progression-free survival; MST: Median survival time; ORR: Objective response rate.

Table 6 Phase Ⅱ studies of S-1 and radiotherapy for locally advanced pancreatic cancer

Ref.	n	ORR	PFS/TTP (mo)	MST (mo)
Sudo *et al*[47]	34	41%	8.7	16.8
Ikeda *et al*[48]	60	27%	9.7	16.2
Shinchi *et al*[49]	50	30%	6.7	14.3
Kim *et al*[50]	25	24%	6.5	12.9

PFS: Progression-free survival; TTP: Time to progression; MST: Median survival time; ORR: Objective response rate.

0.003; median, 3.8 mo *vs* 2.7 mo). A phase Ⅲ study of SL *vs* S-1 alone is now ongoing (GRAPE study).

CHEMORADIOTHERAPY FOR LOCALLY ADVANCED PANCREATIC CANCER

The prognosis of patients with locally advanced pancreatic cancer is dismal with a reported median survival of 6.4 mo if managed with only best supportive care[39]. Chemoradiotherapy (CRT) using 5-FU has been a conventional option in the management of locally advanced pancreatic cancer. The rationale of this combination approach is to control local tumor growth using 5-FU as a radiosensitizer[40]. However, the efficacy of CRT using 5-FU remains limited with a reported median survival time of approximately 10 mo[41,42]. Because distant metastases are the major cause of treatment failure, more effective systemic therapies are necessary to improve patient outcome[42]. In this regard, S-1 is an attractive alternative to 5-FU infusion because it has systemic activity for metastatic or locally advanced pancreatic cancer as shown in the GEST study. Furthermore, a recent preclinical study has demonstrated that gimeracil, a DPD inhibitor included in S-1, enhances antitumor activity of radiotherapy[43].

To date, several schedules of S-1 and concurrent radiotherapy have been investigated in phase Ⅰ/Ⅱ studies for locally advanced pancreatic cancer (Table 6). Sudo *et al*[44] and Ikeda *et al*[45] reported that the standard daily dose of S-1 for systemic chemotherapy (80 mg/m^2 per day) can be combined with radiotherapy (50.4 Gy in 28 fractions). Shinchi *et al*[46] reported that S-1 at a dose of 80 mg/m^2 per day given on days 1 to 21 can be combined with radiotherapy (50 Gy in 40 fractions for 4 wk).

Table 7 Randomized phase Ⅲ studies of adjuvant therapy in pancreatic cancer

Study	Treatment	Endpoint	n	DFS (mo)	MST (mo)
ESPAC-1[55]	CRT (5-FU + RT)	OS	145	10.7	15.9
	No CRT		144	15.2[a]	17.9
	5-FU + leucovorin		147	15.3[c]	20.1[b]
	No chemotherapy		142	9.4	15.5
CONKO-001[56]	GEM	DFS	179	13.4[d]	22.1
	Surgery alone		175	6.9	20.2
JSAP-02[58]	GEM	OS	58	11.4[e]	22.3
	Surgery alone		60	5.0	18.4
ESPAC-3[59]	GEM	OS	537	14.3	23.6
	5-FU + folinic acid		551	14.1	23.0
JASPAC-01[11,12]	GEM	OS	191	11.2	25.9
	S-1		187	23.2[f]	NA[f]

[a]$P < 0.05$ *vs* CRT; [c]$P < 0.05$ *vs* No chemotherapy; [b]$P < 0.01$ *vs* No chemotherapy; [d]$P < 0.01$ *vs* Surgery alone; [e]$P < 0.05$ *vs* Surgery alone; [f]$P < 0.01$ *vs* GEM. DFS: Disease-free survival; MST: Median survival time; NA: Not available; OS: Overall survival; 5-FU: 5-fluorouracil.

Phase Ⅱ studies of S-1 and concurrent radiotherapy have demonstrated an acceptable toxicity profile and promising efficacy with a response rate of 24%-41% and median survival of 12.9-16.8 mo[47-50]. Furthermore, some patients (0%-4%) underwent curative resection after S-1 and radiotherapy in these studies.

Instead of using S-1, capecitabine-based CRT has been reported in Western countries[51,52]. Capecitabine is an oral fluoropyrimidine carbamate, which is converted to 5-FU predominantly in tumor tissues[53]. Saif *et al*[51] reported a phase Ⅱ study of capecitabine and radiotherapy in patients with locally advanced pancreatic cancer with a response rate of 20% and a 1-year survival rate of 58%. A recent randomized phase Ⅱ study of GEM-based or capecitabine-based CRT for locally advanced pancreatic cancer (SCALOP study) has suggested that capecitabine-based CRT might be preferable to GEM-based CRT[54].

ADJUVANT CHEMOTHERAPY FOR RESECTED PANCREATIC CANCER

Adjuvant chemotherapy with GEM has been accepted as the standard treatment in patients with resected pancreatic cancer based on the results of randomized controlled studies (Table 7)[55-59]. In a phase Ⅲ study of adjuvant chemotherapy with GEM *vs* observation in patients with resected pancreatic cancer (CONKO-001), adjuvant GEM significantly improved disease-free survival (median, 13.4 mo *vs* 6.9 mo, $P < 0.001$) compared with the observation group[56]. Adjuvant chemotherapy with GEM improved disease-free survival (median, 11.4 mo *vs* 5.0 mo, $P = 0.01$) in another phase Ⅲ study conducted in Japan (JSAP-02)[58]. The European Study Group of Pancreatic Cancer (ESPAC) conducted a phase Ⅲ study of 5-FU plus folinic acid *vs* GEM following pancreatic cancer resection (ESPAC-3). This study showed no difference in OS between arms (median OS = 23.0 mo *vs*

23.6 mo, $P = 0.39$), but treatment-related serious adverse events were more frequent in patients treated with 5-FU plus folinic acid[59].

Recently, Uesaka *et al*[11] reported on a randomized phase Ⅲ study of GEM *vs* S-1 in patients with pathological stage Ⅰ, Ⅱ or Ⅲ (with celiac axis resection) macroscopically resected (R0 or R1 resection) pancreatic cancer (JASPAC-01)[12]. Between April 2007 and June 2011, 385 patients were randomly assigned to adjuvant GEM ($n = 193$) or S-1 ($n = 192$). The primary endpoint was non-inferiority of S-1 compared to GEM in OS. At the interim analysis, S-1 showed non-inferiority to GEM and, surprisingly, superiority to GEM in OS with a hazard ratio of 0.56 (95%CI: 0.42-0.74; $P < 0.0001$ for non-inferiority; $P < 0.0001$ for superiority). The 2-year survival rates were 53% (95%CI: 46%-60%) for GEM and 70% (95%CI: 63%-76%) for S-1. The quality of life analysis was significantly better in the S-1 arm ($P < 0.0001$). The frequency of grade 3 or 4 toxicities was similar in both arms, except for leukopenia, which was lower in the S-1 arm. The findings of the JASPAC-01 study suggest that adjuvant chemotherapy with S-1 is a more effective alternative to the standard adjuvant chemotherapy with GEM for resected pancreatic cancer.

The rationale for adjuvant chemotherapy lies in eliminating micrometastases and subsequently improving prognosis. The JASPAC-01 study suggests that adjuvant chemotherapy with S-1 achieves better OS and is presumably more effective in eliminating micrometastases. Preclinical studies have suggested that postoperative chemotherapy with S-1 has a moderate effect on eliminating micrometastases[60,61], and the efficacy is higher in smaller micrometastases[61]. As shown in the GEST study, S-1 has significantly higher ORR compared with GEM in patients with metastatic or locally advanced pancreatic cancer. The superior antitumor activity of S-1 might have inhibited micrometastases and resulted in the improvement of OS in an adjuvant setting. Further investigations are necessary to elucidate the basic mechanisms of the efficacy of S-1 in adjuvant chemotherapy. S-1 is also accepted as the standard adjuvant chemotherapy in patients with curatively resected gastric cancer based on a randomized study in Japan[62].

FUTURE PERSPECTIVES

S-1 is now accepted as one of the important key drugs in the management of pancreatic cancer in Japan. Oral administration of S-1 is a convenient option for first-line chemotherapy for metastatic or locally advanced pancreatic cancer because it has shown non-inferiority to GEM in OS. However, there are no definitive criteria for its use instead of GEM, and more aggressive therapies, such as FOLFIRINOX and GEM plus nab-paclitaxel, may be preferable for patients with metastatic pancreatic cancer with good performance status. In adjuvant chemotherapy for resected pancreatic cancer, S-1 is a more effective alternative to the standard chemotherapy with GEM. S-1-

based second-line chemotherapy for GEM refractory pancreatic cancer and CRT using S-1 for locally advanced disease appear to be promising strategies. However, the efficacy of these therapies should be confirmed in future randomized controlled studies.

In reported studies, one of the important features of S-1-based therapy is favorable ORR. Considering this advantage, some investigators are hopeful that S-1-based therapy may be useful in neoadjuvant therapy for potentially resectable or borderline resectable pancreatic cancer. The main goal of neoadjuvant therapy is to downsize tumors and increase the likelihood of curative resection. To date, many studies have evaluated the effectiveness of neoadjuvant CRT or neoadjuvant chemotherapy[63-66]. However, the effectiveness of neoadjuvant therapy still remains controversial, and no standard regimen has been established. In Japan, clinical studies of S-1-based therapies, such as GS therapy or CRT using S-1 in neoadjuvant settings, are now ongoing (*e.g.*, JASPAC-05 and Prep-02/JSAP-05)[67].

In contrast, there are some problems to be resolved with regard to the clinical use of S-1 for pancreatic cancer. Randomized controlled studies of S-1 are conducted mainly in Japanese populations, and it remains unclear if S-1 is also effective in Western populations. Cytochrome P450 2A6 activity is different among ethnic groups[68], which is the key enzyme in converting tegafur to 5-FU[69]. Gastrointestinal toxicity, such as diarrhea, has been reported to be more severe in Caucasian patients[70]. This clinical question should be addressed in future studies. In Western countries, capecitabine, another oral fluoropyrimidine, has been in use in clinical practice, although limited evidence supports its use for pancreatic cancer.

Including S-1, we now have several options in first-line chemotherapy. However, there are no definitive criteria for treatment choice for each patient. In the next step, we should make an effort to develop a predictive biomarker for treatment efficacy. Human equilibrative nucleoside transporter protein expression has been reported to be a possible predictive marker of benefit from adjuvant GEM in patients with resected pancreatic cancer[71,72]. Thymidylate synthase or DPD gene expression levels have been reported as possible predictive markers of the efficacy of adjuvant chemotherapy with S-1 in patients with resected gastric cancer[73]. In the future, understanding of the molecular mechanism of drug sensitivity and cancer pathogenesis is essential to develop personalized cancer treatment. Given the recent advances in molecular biology, further progress in this field is highly expected.

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2014 ADVANCES IN PANCREATIC CANCER

Neoadjuvant therapy for pancreas cancer: Past lessons and future therapies

Jeffrey M Sutton, Daniel E Abbott

Jeffrey M Sutton, Daniel E Abbott, Department of Surgery, Division of Surgical Oncology, University of Cincinnati Medical Center, Cincinnati, OH 45219, United States
Author contributions: Sutton JM and Abbott DE performed research, analyzed data, and wrote the manuscript.
Correspondence to: Daniel E Abbott, MD, Division of Surgical Oncology, Department of Surgery, University of Cincinnati Medical Center, 234 Goodman Street, ML 0772, Cincinnati, OH 45219, United States. abbottdl@ucmail.uc.edu
Telephone: +1-513-5848900 Fax: +1-513-5840459
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Abstract

Pancreatic adenocarcinoma remains a most deadly malignancy, with an overall 5-year survival of 5%. A subset of patients will be diagnosed with potentially resectable disease, and while complete surgical resection provides the only chance at cure, data from trials of postoperative chemoradiation and/or chemotherapy demonstrate a modest survival advantage over those patients who undergo resection alone. As such, most practitioners believe that completion of multimodality therapy is the optimal treatment. However, the sequence of surgery, chemotherapy and radiation therapy is frequently debated, as patients may benefit from a neoadjuvant approach by initiating chemotherapy and/or chemoradiation prior to resection. Here we review the rationale for neoadjuvant therapy, which includes a higher rate of completion of multimodality therapy, minimizing the risk of unnecessary surgical resection for patients who develop early metastatic disease, improved surgical outcomes and the potential for longer overall survival. However, there are no prospective, randomized studies of the neoadjuvant approach compared to a surgery-first strategy; the established and ongoing investigations of neoadjuvant therapy for pancreatic cancer are discussed in detail. Lastly, as the future of therapeutic regimens is likely to entail patient-specific genetic and molecular analyses, and the treatment that is best applied based on those data, a review of clinically relevant biomarkers in pancreatic cancer is also presented.

Core tip: The sequence of multi-modality therapy for pancreatic cancer continues to be debated, though many pancreatic cancer specialists are increasingly utilizing neoadjuvant chemoradiation prior to surgical resection. This manuscript details the rationale for neoadjuvant therapy, the data that supports its use, and the potential of biomarker use for personalizing care in pancreatic cancer.

INTRODUCTION

The diagnosis of pancreatic cancer portends a very poor prognosis. Worldwide, approximately 300000 new cases of pancreatic cancer are diagnosed annually, while in the United States, pancreatic adenocarcinoma remains the fourth leading cancer-related cause of death in both men and women[1]. The American Cancer Society estimates that approximately 45000 patients will be diagnosed with pancreatic cancer in 2013 while over 37000 patients will succumb to this disease by year's end[2]. The average American's lifetime risk for developing pancreatic cancer

is 1%-2% and, unlike most other malignancies, the incidence of pancreatic cancer has been slowly increasing over the last decade. Pertinent risk factors for developing pancreatic cancer include chronic pancreatitis, smoking, diabetes mellitus, significant family history, and certain genetic disorders such as cystic fibrosis, hereditary pancreatitis, Peutz-Jeghers syndrome, and Lynch syndrome[3]. Despite more sophisticated imaging modalities including high-resolution computed tomography scanning and endoscopic ultrasound, the overall 5-year survival for all patients with pancreatic cancer approaches 5%. This abysmal statistic is underscored by the fact that many patients present late in their disease process, with four out of five patients initially presenting with unresectable tumor burden; one-third of these patients will have locally-advanced disease deemed unresectable while the remaining two-thirds will have evidence of stage IV distant metastases found on staging work-up[4].

Complete resection of pancreatic cancer with negative surgical margins is obligatory for long-term survival, and, while surgery remains the only curative therapy for pancreatic cancer, only 15% of patients undergoing resection are actually cured of their disease long-term[5]. The remaining 85% will eventually develop locoregional recurrence and/or distant metastases. Frequently, pancreatic cancer recurs systemically while sparing the surgical site, suggesting systemic disease was present at the time of diagnosis and initial resection. As such, both chemoradiation and chemotherapy have been utilized as adjuncts to surgical resection in an attempt to minimize locoregional and distant recurrence, respectively.

Two major drivers of poor survival in patients with pancreatic cancer are delayed detection and lack of effective chemotherapy. Unlike many other gastrointestinal malignancies, pancreatic cancer tends to remain asymptomatic and undiagnosed until significant locoregional or distant disease is present. While early detection is rare, patients diagnosed with tumors limited to the pancreas without nodal involvement who undergo resection can experience a median survival of 33 mo with 1-, 3-, and 5-year survivals of 80%, 49%, and 41%, respectively[6]. Unfortunately, current screening modalities are neither sensitive nor specific enough to identify and diagnose these tumors at such an early stage, limiting the number of patients who undergo meaningful and curative surgical resection. Furthermore, current chemotherapy regimens are only marginally effective in extending survival (a fact that has not changed in over 30 years)[7-12]. Based on these challenges, recent attention has turned to novel therapeutic agents and delivery sequences in an attempt to improve survival within this vulnerable patient population.

LESSONS FROM THE PAST - ADJUVANT THERAPY TRIALS

Several prospective randomized trials investigating the benefit of adjuvant therapy following surgical resection paved the way for current practice patterns. Early studies utilized fluorouracil (5-FU) as the backbone of chemoradiation and/or chemotherapy regimens. In 1985, the Gastrointestinal Tumor Study Group (GITSG) demonstrated a nearly 2-fold improvement in median survival and a 3-fold improvement in 2-year survival rates of patients who received systemic 5-FU and 5-FU based chemoradiation (40 Gy) following resection[7]. However, due to the small sample sizes (*n* = 22 for observation, *n* = 21 for treatment), as well as the historically low survival within the control group, many have remained critical of these early findings. Fourteen years later, the European Organisation of Research and Treatment of Cancer (EORTC) published a study comparing split course 5-FU-based chemoradiation *vs* observation following resection with curative intent of combined pancreatic and periampullary tumors[13]. Despite the modest improvement in survival (17.1 mo *vs* 12.6 mo) in patients who received adjuvant therapy, the study was underpowered to achieve statistical significance in the subset of patients with pancreatic tumors, and these authors concluded that chemoradiation was not beneficial in this setting.

In an effort to further clarify the role of adjuvant therapy, the European Study Group for Pancreatic Cancer (ESPAC) investigated the efficacy of both chemotherapy and chemoradiation in patients undergoing surgical resection[9]. Utilizing a 2 × 2 factorial design, patients were randomized to either observation, 5-FU based chemotherapy, 5-FU based chemoradiation, or both after undergoing curative resection. This study demonstrated a significant survival benefit for patients who received systemic 5-FU based chemotherapy, while chemoradiation conferred a negative prognosis, leading to a European standard of care that does not include radiation therapy in the multimodal treatment of pancreatic cancer. Due to criticisms regarding the randomization scheme, the relatively low-dose of radiation administered (a hypofractionated dose of 20 Gy *vs* the traditional 50.4 Gy), as well as the lack of quality control of the radiotherapy administered, the negative prognostic effect of chemoradiotherapy was generally not accepted within the United States.

For many years, 5-FU was the only efficacious chemotherapeutic agent available in the treatment of pancreatic cancer. One promising alternative, gemcitabine, was introduced in the 1990s, and initial phase 1 studies demonstrated a reasonable safety profile with low rates of significant toxicity[14]. Burris *et al*[15] noted that patients with locally advanced pancreatic cancer treated with gemcitabine demonstrated a significant clinical benefit and modest improvement in survival compared to treatment with the traditional 5-FU regimen. That same year, the phase 3 study CONKO-001 began accruing. This trial sought to compare adjuvant single-agent gemcitabine *vs* observation in patients following curative-intent resection of pancreatic cancer. Almost a decade later, the initial results of the CONKO-001 trial demonstrated a significantly prolonged disease-free survival in the

gemcitabine arm[8]. An updated analysis published this year with a median follow-up of 136 mo confirmed the disease-free survival advantage of gemcitabine compared to observation (13.4 mo *vs* 6.7 mo, *P* < 0.001). Beyond that, patients treated with adjuvant gemcitabine also had a significantly improved overall survival (OS). Five- and ten-year OS rates between the gemcitabine and observation groups were 20.7% *vs* 10.4% and 12.2% *vs* 7.7%, respectively[16]. In a more recent phase III trial, the US Intergroup/RTOG9704 study investigated the impact of adding gemcitabine to a 5-FU based chemoradiation and chemotherapy regimen following resection[17]. This study demonstrated a modest clinical (but not statistically significant) improvement in median and 5 year overall survivals. These adjuvant trials, and other studies substantiating the activity of gemcitabine in the setting of advanced and metastatic disease, have established this drug as a standard first-line therapy postoperatively[18].

RATIONALE FOR NEOADJUVANT THERAPY

While chemoradiation and systemic chemotherapy have been shown to improve survival in patients undergoing surgical resection of pancreatic cancer, the sequencing of these treatment modalities remains a topic of continued research and debate. Specifically, with emerging data that chemotherapy and/or chemoradiation prior to surgical extirpation can be associated with superior patient outcomes, the notion of neoadjuvant therapy followed by resection has gained traction among many pancreatic cancer specialists. As the neoadjuvant strategy evolves, the question of which patients should undergo neoadjuvant therapy persists. Should all patients with potentially resectable tumors receive chemoradiation prior to surgery, or only those with borderline resectable or locally advanced tumors? And which patients are likely to benefit most from a neoadjuvant regimen? Currently, we do not have strong evidence-based answers to these questions. However, supporters of a neoadjuvant approach point to its ability to select a patient population that will ultimately and maximally benefit from completion of multimodality therapy. In general, initially treating patients with a neoadjuvant approach will inherently "select-in" ideal candidates for surgery while "selecting-out" poor operative candidates or those with distant disease[19]. A more detailed rationale for the neoadjuvant approach is outlined here (Table 1).

Improving margin negative resection rates
Neoadjuvant radiotherapy results in the killing of cancer cells at the periphery of the tumor. By sterilizing the microscopic edge of the tumor, patients may experience improved negative margin resection rates and therefore a reduction in local recurrence[20,21]. Because surgical resection leads to a significant disruption of the native blood supply within the pancreatic tissue and neighbor-

Table 1 Rationale for patient selection for neoadjuvant therapy in pancreatic cancer

Rationale in support of neoadjuvant therapy
Increasing the likelihood of margin-negative resection[4,20-25]
Increasing the likelihood of completion of multimodality therapy[5,26-29]
Increasing efficacy of radiotherapy[4,24]
Minimizing pancreatic leak (without increasing complications)[19,30-34]
Determination of indeterminant lesions[29,35]
Declaration of distant metastases[4,29]
Decreasing "open-and-close" rates[19]
Allowing a patient's functional status to declare itself[36]
Improved cost-effectiveness[37]

ing retroperitoneal nodal basin, chemotherapeutic agents delivered as pro-drugs which rely on the production of active metabolites for cytotoxicity and/or sensitization of the tissue prior to radiation may not be delivered as effectively to the site of cancer in post-operative tissue without an intact blood supply[22]. A study published in *Annals of Surgery* in 2008 investigated the tissue-level response in patients with resectable pancreatic tumors undergoing neoadjuvant chemotherapy[23]. In this prospective phase II trial, patients received 4 bi-weekly cycles of gemcitabine and cisplatin prior to restaging and surgical resection. Following therapy, cytopathologic and histological responses to the chemotherapy regimen were noted in 83% and 54% of patients, respectively. A significant reduction in tumor metabolism as determined by fluorodeoxyglucose uptake was also identified as compared to baseline, and this finding correlated with histological Ki-67 expression. Similarly, external beam radiotherapy requires well-perfused and well-oxygenated tissue to exert its maximal ionizing damage. Relative tissue hypoxia has been shown to confer radiation resistance, especially in an adjuvant setting[4,24]. By providing radiotherapy to unadulterated and well-oxygenated tissue, radiation therapy proves more potent and efficacious. Furthermore, receipt of radiotherapy preoperatively allows for delivery of a smaller dose in a more directed radiation field and avoids administering radiation to a freshly reconstructed bowel anastomosis[4].

Increasing resectability
While pre-operative locoregional and systemic therapy has the potential to improve rates of margin-negative resection, neoadjuvant therapy can also increase the number of operable candidates by converting an initially unresectable tumor to a resectable one. A meta-analysis by Gillen *et al*[25] demonstrated that approximately one-third of patients who were deemed unresectable at initial staging may undergo neoadjuvant therapy and convert to operable candidates while maintaining similar survival estimates as those initially deemed resectable. With the estimated 45000 new cases diagnosed annually it the United States, this translates to a chance at a curative resection for 15000 patients who would have otherwise died of their disease burden.
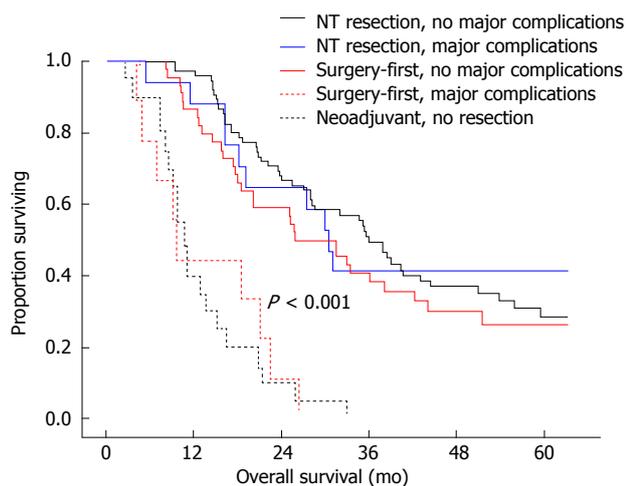
Figure 1 Survival in patients with pancreatic cancer who undergo neoadjuvant therapy *vs* a surgery-first approach. Survival curves for patients who complete multimodality therapy, inclusive of chemoradiation and curative-intent resection, incorporating major postoperative complications or those who never undergo resection.


Figure 2 Outcomes in patients who undergo neoadjuvant therapy. Of those patients with potentially resectable pancreatic tumors who undergo chemoradiation, 71% went on to undergo complete surgical resection. Reasons for not undergoing complete resection included declaration of distant metastases, prohibitive performance status, anatomically unresectable locally-advanced tumor, and patient refusal.

Completion of multimodality therapy

The use of multimodality therapy (surgical resection, chemotherapy and radiation therapy) is being increasingly recognized as the optimal approach to treating patients with pancreatic cancer[26]. However, the efficacy of multimodality therapy, whether given pre- or postoperatively, is contingent upon completion of the ascribed regimen. Given the inherent morbidity and associated post-operative complications of pancreatic surgery, an estimated 25%-50% of patients will experience a delay or never initiate adjuvant therapy[5,27,28]. A recent study from The University of Texas MD Anderson Cancer Center evaluated the rates of completion of multimodality therapy in those who underwent neoadjuvant therapy *vs* those who initially underwent surgical resection[29]. Eighty-three percent of patients who underwent neoadjuvant therapy received their entire multimodality regimen *vs* just 58% of patients who underwent surgical resection first. Consider also that 100% of patients who underwent resection - the modality of pancreatic cancer treatment proven to matter the most - received the maximal benefit of their multimodality therapy. While there was no significant difference in major postoperative complications between the two treatment strategies, those patients who experienced major postoperative complications were less likely to complete their adjuvant multimodality therapy. Not surprisingly, those patients who completed multimodality therapy demonstrated a significant survival advantage compared to those who did not (36 mo *vs* 11 mo, *P* < 0.001) regardless of timing (Figure 1). This study demonstrates that patients who undergo neoadjuvant therapy are more likely to not only initiate but also complete their course of non-surgical treatment, and therefore experience its full therapeutic potential.

Minimizing pancreatic leak (without increasing complications)

As directed radiation can result in significant glandular fibrosis, the radiation therapy itself may allow for a pancreatic anastomosis with a lower leak rate as a result of a firmer, fibrotic pancreas[30-32]. Numerous studies evaluating intra- and post-operative complications following neoadjuvant chemoradiation have noted relative reductions in pancreatic anastomotic leak rates in patients treated preoperatively, with pancreatic fistula rates in the single digits[19,33]. Though some studies have demonstrated that neoadjuvant therapy is associated with higher intraoperative blood loss, vascular reconstruction rates, and longer operative time, no documented studies have demonstrated increased rates of postoperative complications in patients following neoadjuvant therapy[31,34].

Indeterminant lesions, declaration of distant metastases, and decreasing "open-and-close" rates

Roughly half of the patients who are newly diagnosed with pancreatic adenocarcinoma present with distant metastases at the time of initial staging[4]. Additionally, a significant number of patients without evidence of distant metastases on initial staging are found to have unanticipated metastases at the time of surgery (though this is partially dependent upon the interval duration between imaging and operation); these patients likely harbored micrometastatic tumor cells at the time of initial staging[35]. As such, neoadjuvant therapy may help to identify this subset of patients and prevent "open-and-close" operations. Still yet, some patients may demonstrate no evidence of distant disease on initial staging or at the time

of surgery, yet develop radiographic evidence of distance metastases within months of resection. Undergoing neoadjuvant treatment in this scenario provides time for micrometastases to declare themselves radiographically, prior to operation. A recent study by Tzeng *et al*[29] outlined these possibilities (Figure 2). In those patients who demonstrate distant disease at the completion of neoadjuvant therapy, the identification of aggressive tumor biology spares them the morbidity of an otherwise futile surgery. An additional cohort of patients may have indeterminate lesions on initial staging. Neoadjuvant treatment allows for these potentially metastatic lesions to enlarge, shrink, or remain unchanged-thus providing additional diagnostic information for otherwise potentially resectable disease prior to undergoing surgery.

Temporal assessment of functional status

In addition to those patients with anatomically borderline pancreatic tumors, the group from MD Anderson describes another cohort of patients who may have a borderline functional status. This group, termed Borderline Resectable Type C, are those patients with resectable tumors who present as sub-optimal surgical candidates given their extreme age, poor functional status, significant weight loss and/or malnutrition, or debilitating medical comorbidities[36]. Utilizing the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) Database, they noted that approximately one-third of patients undergoing elective pancreaticoduodenectomies met their criteria for Borderline Type C[36]. Moreover, this cohort was more likely to experience major postoperative complications and death compared to medically-optimized patients[28]. One may suggest that neoadjuvant therapy in these frail patients could provide a window for medical optimization prior to surgery, or at the very least a chance for a debilitated functional status to declare itself prior to undergoing the morbidity of a pancreatic resection.

Cost-effectiveness

In addition to data supporting neoadjuvant therapy from a patient outcomes perspective, there are data to suggest that neoadjuvant therapy is more cost-effective -from a societal perspective - than a surgery first approach. A recent study by Abbott *et al*[37] utilized data from the National Surgical Quality Improvement program, the American College of Surgeons National Cancer Data Base, and a prospectively-maintained database of patients undergoing neoadjuvant therapy at MD Anderson to construct an analytic model investigating the costs and survival for patients undergoing various treatment strategies. The authors concluded that receipt of neoadjuvant chemoradiation for pancreatic cancer yielded an improved survival (reported in quality-adjusted life months) as well as a significant cost savings of approximately $10000 per patient-case compared to those undergoing a surgery-first approach. In our current healthcare climate, which stresses improved quality under continued fiscal constraints, treatment strategies that achieve optimal outcomes at reduced costs will be increasingly expected.

Limitations of neoadjuvant therapy

There are, of course, limitations to pursuing neoadjuvant therapy in all patients. Firstly, patients with initially resectable tumor burdens may experience local progression of their disease while receiving neoadjuvant therapy. Though very rare (2%-3%) in these patients, the absence of distant metastases with concomitant local advancement may result in an unresectable tumor. In these patients, a surgery-first approach may have benefitted them, though with an aggressive tumor biology - marked by progression on chemotherapy - it is difficult to make any strong conclusions about outcomes in this small cohort of patients. Secondly, unlike data on adjuvant therapy regimens, large randomized prospective phase III trials investigating the efficacy of neoadjuvant regimens are lacking. To date, no phase III trials directly comparing neoadjuvant therapies to adjuvant therapies have been published; as such, we are currently forced to extrapolate our knowledge from smaller phase I / II investigations.

NEOADJUVANT THERAPY TRIALS

Two recently published reviews by Lowy[5] and Abbott *et al*[38] extensively discuss the landmark neoadjuvant trials published through 2008. Here, we will review a select few of these studies and proceed to focus on recent investigations published within the last 5 years (Table 2).

The first major study investigating the effects of chemoradiation in the neoadjuvant setting was published in 1993 by Yeung *et al*[39]. In this Phase II study, patients with biopsy-proven pancreatic (*n* = 26) or duodenal cancer (*n* = 5) were treated with 50.4 Gy of radiation and concurrent tissue-sensitizing 5-FU and mitomycin C. Due to progression of disease in one-third of the study population, patients with pancreatic adenocarcinoma achieved a resection rate of 38%. In those resected patients, the authors reported a reduction in the rate of positive surgical margins and regional lymph node involvement in patients who underwent neoadjuvant therapy. The 5-year survival rates were 58% and 0% for those patients who underwent resection *vs* those unresected, respectively.

Since that initial study, further investigations into the role of neoadjuvant therapy prior to attempted resection have demonstrated improved resection rates with variability in effects on median survival. With various radiation regimens (30.0 to 50.4 Gy), combinations of chemotherapeutic agents (*e.g.*, 5-FU, mitomycin C, paclitaxel, and cisplatin) and dosing, resection rates ranging from 45%[27] to 85%[40] were associated with median survival durations of two years or less. A recent Phase II study from France published in 2008 enrolled 41 patients with localized, potentially resectable pancreatic adenocarcinoma and treated them with 50 Gy of radiation combined with 5-FU and cisplatin[41]. Twenty-six patients (63%) went on to curative resection, and 81% had an R0 resec-

Table 2 Recently published prospective neoadjuvant trials of multimodal therapy for pancreatic cancer *n* (%)

Ref.	Initial staging	Regimen		Resection	Survival	Notes
		Chemoradiation	Chemotherapy			
Palmer *et al*[106], 2007	Resectable (*n* = 50)	N/A	Gem *vs* Gem + Cis	Overall: 27 (54); Gem: 9 (38), Gem + Cis: and 18 (70)	Gem: 42%, Gem + Cis: 62% (1 yr survival)	Randomized Phase Ⅱ; No difference in surgical complcations
Le Scodan *et al*[41], 2008	Resectable (*n* = 41)	50 Gy + 5-FU + Cis	N/A	26 (63)	Overall: 9.4 mo, R: 11.7 mo (2 yr survival 32%) UR: 5.7 mo	Phase Ⅱ; 67.5% were successfully treated with entire radiation dose and ≥ 75% chemotherapy dose; 81% achieved an R0 resection
Heinrich *et al*[107], 2008	Resectable (*n* = 28)	N/A	Gem + Cis	26 (93)	Overall: 26.5 mo; R: 19.1 mo	Phase Ⅱ; 80% achieved an R0 resection
Evans *et al*[19], 2008	Resectable (*n* = 86)	30 Gy + Gem	N/A	64 (74)	Overall: 22.7 mo; R: 34 mo, UR: 7 mo ($P < 0.001$);	Phase Ⅱ; 27% 5 yr OS, 36% *vs* 0% for resected *vs* unresected
Varadhachary *et al*[33], 2008	Resectable (*n* = 90)	30 Gy + Gem + Cis	Gem + Cis	52 (66)	Overall: 17.4 mo; R: 31 mo, UR: 10.5 mo	Phase Ⅱ
Turrini *et al*[108], 2010	Resectable (*n* = 34)	45 Gy + Docetaxel	N/A	17 (50)	R: 32 mo	Phase Ⅱ; 10% R0 resection; 5 yr survival resected 41%
Landry *et al*[109], 2010	Borderline	Arm A: 50.4 Gy + Gem (*n* = 10)	Arm B: Gem + Cis + 5-FU then 50.4 Gy + 5-FU (*n* = 11)	A: 3 (30) B: 2 (22)	R: 26.3 mo A: 19.4 mo B: 13.4 mo	Phase Ⅱ; early termination due to poor accrual
Sahora *et al*[45], 2011	Unresectable (*n* = 18), borderline (*n* = 15)	N/A	Gem + Oxaliplatin	13 (39)	R: 22 mo UR: 12 mo ($P = 0.046$)	Phase Ⅱ; 69% R0 resection
Sahora *et al*[46], 2011	Borderline (*n* = 12), Unresectable (*n* = 13)	N/A	Gem + Docetaxel	8 (32)	R: 16 mo, UR: 12 mo	Phase Ⅱ; 87% R0 resection
Pipas *et al*[43], 2012	Resectable (*n* = 4), Borderline (*n* = 23), Unresectable (*n* = 6)	54 Gy + Cetuximab + Gem	N/A	25 (76)	R: 24.3 mo	Phase Ⅱ; 92% R0 resection
Wo *et al*[51], 2013	Resectable (*n* = 10)	Short-course photon RT (3 Gy × 10, 5 Gy × 5 qod, 5 Gy × 5 qd) + Capecitabine	N/A	N/A	N/A	Phase Ⅰ; closed early due to intraoperative complications (fibrosis)
Kim *et al*[44], 2013	Resectable (*n* = 23), Borderline (*n* = 39), Unresectable (*n* = 6)	30 Gy + Gem + Oxaliplatin	N/A	43 (63)	Overall: 18.2 mo; R:27.1 mo, UR: 10.9	Phase Ⅱ, multi-institutional
Shinoto *et al*[52], 2013	Resectable (*n* = 26)	30.0-36.8 Gy E of Carbon-ion radiotherapy (CIRT)	N/A	21 (81)	Overall: 42%, R: 52% (5 yr survival)	Phase Ⅰ; short course radiation

Gem: Gemcitabine, Cis: Cisplatin; 5-FU: Fluorouracil; Gy: Gray; R: Resected patients; UR: Unresected patients; N/A: Not available.

tion. Despite this, the 2-year overall survival rate in those resected patients was 32%.

The introduction of gemcitabine-based chemoradiation into neoadjuvant treatment regimens showed more promise for patients with pancreatic adenocarcinoma. In part due to gemcitabine's potent radiosensitizing effect compared to alternative chemotherapeutic agents[42], its use in the neoadjuvant setting has led to significantly longer median survival rates compared to 5-FU, cisplatin, and mitomycin C based chemoradiation regimens, often with an improved side-effect profile. In a recent report from MD Anderson, patients with potentially resectable stage Ⅰ/Ⅱ pancreatic adenocarcinoma received gemcitabine-based chemoradiation with rapid-fractionation external beam radiation therapy (30 Gy)[19]. Of the 74% patients who underwent successful resection, the median survival was 34 mo (compared to 7 mo for the 26% of patients who did not undergo resection). This study

concluded that even with a rapid-fractionation protocol of gemcitabine-based chemoradiation, similar (if not improved) survival outcomes can be achieved in patients who receive the standard-fractionation external beam radiation therapy dose of 50.4 Gy. That same year, the same group failed to demonstrate any benefit of adding gemcitabine and cisplatin to preoperative gemcitabine-based radiation therapy beyond that achieved by neoadjuvant gemcitabine-based radiation alone[33]. However, despite no statistical survival benefit, this trial demonstrated an excellent overall survival of over 30 mo in those undergoing resection - an improvement over many prior trials.

In reviewing these survival data, recognizing the results based on intent-to-treat analyses is critical. Regimen crossover, treatment-related toxicity, debilitating performance status, progression of disease while receiving therapy, patient refusal, and patient death are several factors that prevent patients from receiving the full extent of their therapy. When analyzing all patients with initially potentially resectable pancreatic cancer from these two studies - combining those who underwent resection and those patients who did not - an overall survival of 22.7 and 17.4 mo, respectively, was noted. Based on these intent-to-treat analyses, neoadjuvant therapy followed by resection of the pancreatic tumor yields comparable survival with patients randomized to undergo resection followed by adjuvant therapy. At the very least, neoadjuvant therapy does not result in inferior survival, while sparing a significant number of patients the morbidity of a futile PD. Thus, neoadjuvant therapy should be considered as a favorable therapeutic approach to patients with potentially resectable pancreatic cancer.

Gemcitabine has also been studied in the setting of borderline and unresectable tumors. Pipas *et al*[43] recently investigated a cetuximab/gemcitabine/intensity-modulated radiotherapy combination in an initial cohort consisting of patients with resectable ($n = 4$), borderline resectable ($n = 23$), and unresectable ($n = 6$) tumors. In total, following neoadjuvant therapy, 25 patients (76%) underwent curative-intent resection. Ninety-two percent of resected patients had a negative surgical margin, and two experienced complete pathologic responses to the regimen. The median survival of those patients who underwent resection was 24.3 mo, comparable to historical controls of patients initially deemed resectable upon presentation. A similar finding was recently published by Kim *et al*[44], evaluating gemcitabine, oxaliplatin, and 30 Gy of radiation in patients with localized and locally-advanced pancreatic adenocarcinoma. Two-thirds of the 68 enrolled patients initially presented with either borderline or unresectable tumors. However, 63% of patients went on to curative resection, and the median survival of patients undergoing resection was 27.1 mo.

Others have investigated neoadjuvant chemotherapy without concurrent radiation therapy. Sahora *et al*[45] have reported two phase II trials evaluating gemcitabine-based neoadjuvant chemotherapy alone in patients with borderline or unresectable tumors. One study used a NeoGemOx protocol consisting of gemcitabine and oxaliplatin given as IV infusions once weekly for 6-9 wk[45]. Notably, 18 patients presented with disease deemed unresectable at inclusion, while the remaining 15 patients had borderline resectable tumors. Following treatment with the NeoGemOx regimen, 13 patients, or 39% of those without clearly resectable tumors proceeded to undergo resection with curative intent. The median overall survival of this cohort was 22 mo, statistically longer than those without significant tumor regression. The second study utilized gemcitabine and docetaxel-based neoadjuvant chemotherapy (NeoGemTax) in a similar patient population[46]. Of the total 25 eligible patients, 13 had unresectable disease and 12 had borderline resectable disease at the time of inclusion. Although a similar percentage of patients (32%) experienced tumor regression to allow an attempt at curative resection as compared to the NeoGemOx regimen, the median survival of those patients downstaged with the NeoGemTax did not significantly differ from those patients whose tumors failed to regress (16.3 mo *vs* 12.2 mo). Despite a lack a radiotherapy in either study, R0 resection rates for the NeoGemOx and NeoGemTax regimens were 69% (9/13 resected patients) and 87% (7/8 patients), respectively. It is important to keep in mind, however, the relatively low sample sizes within these phase II trials ($n = 33$ and $n = 25$) as well as the overall resection rates (13/33, 39%; and 8/25, 32%) before concluding that radiation has a limited role in the neoadjuvant setting.

THERAPIES OF THE FUTURE

Ongoing trials

In an attempt to better define optimal treatment sequences, the NEOPAC study (NCT01521702) is an ongoing multicenter prospective randomized phase III trial which aims to determine the efficacy of neoadjuvant chemotherapy *vs* adjuvant chemotherapy in patients with pancreatic head cancer[47]. This trial is currently recruiting patients with biopsy-proven resectable cancer who will be randomized to one of two arms: (1) neoadjuvant chemotherapy consisting of gemcitabine and oxaliplatin followed by surgery and adjuvant gemcitabine; or (2) initial surgical resection followed by adjuvant gemcitabine, with the primary endpoint of progression-free survival. No patients are randomized to receive concurrent radiotherapy, as this study aims to investigate the efficacy of chemotherapy only.

The Interdisciplinary Study Group of Gastrointestinal Tumours of the German Cancer Aid is also currently performing a multicenter randomized phase II trial comparing neoadjuvant therapy with adjuvant therapy in patients undergoing resection of their pancreatic tumor[48]. Patients with potentially resectable tumors are randomized to A) neoadjuvant chemoradiation with concurrent gemcitabine and cisplatin followed by surgical resection or B) immediate resection. Given its repeated demonstration of therapeutic benefit, post-operative adjuvant chemotherapy will be administered for 6 mo to patients in

both arms of the study.

The combination chemotherapeutic regimen of 5-FU, leucovorin, irinotecan, and oxaliplatin, referred to collectively as FOLFIRINOX, is a newer treatment option for patients with pancreatic cancer. A recent phase III study published in the *New England Journal of Medicine* found this regimen to be superior to the gemcitabine in patients with metastatic pancreatic cancer, with prolonged median overall survival (11.1 mo *vs* 6.8 mo) and disease-free survival (6.4 mo *vs* 3.3 mo)[11]. Given the relative success of this novel regimen in the metastatic setting, the Alliance for Clinical Trials in Oncology is spearheading a multicenter single-arm pilot study (Alliance A021101) to evaluate FOLFIRINOX as a neoadjuvant regimen for patients with borderline resectable tumors at initial presentation, utilizing FOLFIRINOX followed by 50.4 Gy of capecitabine-based chemoradiotherapy prior to surgery[49]. Primary outcomes are focused on survival and toxicity of this regimen. This will be the first multicenter trial specifically evaluating FOLFIRINOX in the neoadjuvant setting for patients with borderline resectable disease.

For those patients with locally advanced unresectable pancreatic cancer, the RECLAP trial is a phase I study investigating the safety and efficacy of super-selective intra-arterial delivery of chemotherapy to the tumor bed *via* an indwelling subcutaneous port[50]. Outcomes of interest include toxicity, disease-free and overall survival, and conversion from unresectable to potentially resectable tumors.

Wo *et al*[51] recently published a small phase I study of patients with resectable pancreatic cancer who underwent neoadjuvant accelerated short-course photon chemoradiation therapy with concurrent capecitabine. Patients received photon radiotherapy at escalating doses of 3Gy × 10 d, 5 Gy × 5 d administered every other day, and 5 Gy × 5 consecutive days. Unfortunately, this radiation protocol resulted in significant intraoperative morbidity associated with radiation-induced fibrosis of the surgical field and forced the study to close early. Shinoto *et al*[52] investigated the toxicity and efficacy of carbon-ion radiotherapy (CIRT) as a short-course neoadjuvant treatment in patients with resectable tumors. The dose of CIRT was sequentially increased by 5% increments from 30 to 36.8 Gy equivalents with resection 2 to 4 wk after the completion of CIRT. None of the resected patients experienced local recurrence, and 5-year survival rates for those resected was 52% - a rather promising finding when compared to historical rates.

Finally, the NEOPANC trial is a single-arm prospective phase I / II study investigating neoadjuvant short course intensity-modulated radiation therapy in combination with surgery and intraoperative radiation therapy of 15 Gy for the treatment of resectable pancreatic cancer[53]. The authors hypothesize that neoadjuvant and intraoperative radiation administration will allow for dose escalation, reduced toxicity, and improved patient tolerance. The primary outcomes include 1 year local recurrence as well as feasibility of delivering such a regimen, with secondary endpoints of overall and disease-free survival, toxicity, and associated morbidity and mortality.

Additional therapeutic options

The novel agent S-1, an oral fluoropyrimidine analogue, has shown great therapeutic success in numerous Japanese studies. S-1 consists of a combination of three drugs: the 5-FU prodrug tegafur, 5-chloro-2,4-dihydroxypyridine (CDHP; an inhibitor of dihydropyrimidine dehydrogenase enzyme activity), and potassium oxonate (OXO; an inhibitor of 5-FU phosphorylation in the gastrointestinal tract, thereby reducing side effects)[54]. This drug mimics the anticancer agent 5-FU by intercalating itself into actively-synthesizing strands of DNA and causing the rapidly dividing cells to undergo apoptosis, and this formulation has clear advantages over 5-FU. The bioavailability of single-agent 5-FU administered orally is minimal due to the high activity of dihydropyrimidine dehydrogenase within the enterocytes, which lead to premature metabolism. CDHP and OXO within S-1 act to prevent the premature metabolism of the prodrug until it has successfully been absorbed and delivered to its target cells. The oral administration is therefore not only more convenient than the IV form of 5-FU, but also allows for predictable absorption and pharmacokinetic properties[54].

Given its widespread success in numerous phase II trials, S-1 was studied in a phase III trial in patients with locally advanced or metastatic disease[55]. This trial was powered to demonstrate non-inferiority of S-1 to gemcitabine, and 834 chemotherapy-naïve patients were randomly assigned to receive S-1, gemcitabine, or both. The noninferiority of S-1 to gemcitabine was demonstrated, but gemcitabine plus S-1 was not superior to gemcitabine alone, providing an argument for the use of S-1 as single-agent therapy in the setting of advanced pancreatic cancer. From these data, a study was designed to evaluate the efficacy of S-1 in the neoadjuvant setting. Tajima *et al*[56] retrospectively evaluated neoadjuvant gemcitabine plus oral S-1 in patients with potentially resectable pancreatic cancer. Of the 13 patients who received the neoadjuvant treatment, no patients demonstrated disease-progression or distant metastases prior to resection. The investigators found a trend towards improved 3-year survival (55.6% for the neoadjuvant treatment group *vs* 29.6% in the resection group), but this pilot study was underpowered to detect a significant difference. A larger phase I study of the gemcitabine plus S-1 neoadjuvant regimen is currently underway. While most trials involving S-1 have been conducted in Japan, additional trials are being developed across Europe and the United States.

Molecular markers of interest

The armamentarium of chemotherapeutic agents used to treat pancreatic cancer in the neoadjuvant setting, though continually improving, is sub-optimal. Prolonging survival by mere months suggests that pharmacological improvements can-and need to-be made. A topic of great interest and ongoing research in the field of chemotherapeutic optimization centers on evolving from a one-size-fits-all

Table 3 Select clinically valuable pancreatic cancer biomarkers			
Gene product	Target drug	Mechanism	Ref.
hENT1	Gemcitabine	Nucleoside inhibitor, prevents DNA synthesis in cancer cells	[59-64]
TS/DPD	5-FU/S-1	Suicide inhibitor of TS, prevents DNA synthesis in cancer cells	[65-71]
EGFR	Erlotinib	Tyrosine kinase inhibitor, prevents EGFR-mediated cell cycle progression and cellular proliferation	[72-79]
CA 19-9	N/A	N/A	[80-88]
SPARC	Nab-paclitaxel	Disruption of microtubule formation during mitosis	[12,89-94]
SMAD4	N/A	Tumor suppression, initiation, and metastasis	[95-99]

CA 19-9: Carbohydrate antigen 19-9; TS: Thymidylate synthase; hENT1: Human equilibrative nucleoside transporter-1; SPARC: Secreted protein acidic and rich in cysteine; EGFR: Epidermal growth factor receptor.

neoadjuvant regimen to a more individualized regimen tailored to a patient's specific tumor genotype and biology. Identifying novel biomarkers associated with pancreatic cancer may not only allow for the development of individualized treatment regimens, but also may allow for earlier disease detection, and an extraordinary amount of research is being performed to identify an accurate tumor marker or a panel of markers that could aid in the management of this disease[57]. As a testament to the current popularity of biomarker identification within pancreatic adenocarcinoma, a recent analysis by Harsha *et al*[58] identified over 2500 gene products with evidence of overexpression at the mRNA level, protein level, or both. Here we will review a select few biomarkers, focusing on those with potential prognostic value and those that may prove "actionable" in future therapeutic endeavors (Table 3).

Human equilibrative nucleoside transporter-1

Gemcitabine is an analog of the nucleoside deoxycitidine. This prodrug is transported into a cell and subsequently phosphorylated to its active forms gemcitabine diphosphate or gemcitabine triphosphate[59]. The active forms of gemcitabine then confer their cytotoxic effects by inserting into synthesizing DNA chains and disrupting further DNA synthesis, and gemcitabine's cellular uptake is dependent upon the human equilibrative nucleoside transporter-1 (hENT1) protein[60]. As this receptor is traditionally upregulated on the surface of pancreatic adenocarcinoma cells, gemcitabine has proven to be an effective chemotherapeutic option in many patients.

However, despite its therapeutic benefit in patients with both resectable and unresectable pancreatic tumors, not all patients respond to gemcitabine treatment. One potential mechanism for resistance to gemcitabine includes a relative downregulation or mutation of hENT1 receptors which ultimately leads to decreased cellular uptake of the drug[60]. *In vitro* analyses of pancreatic tumor cell lines have demonstrated that hENT1 protein expression is a significant determinant of gemcitabine activity

within pancreatic tumor cells: overexpression of the hENT1 protein on the pancreatic cell surface correlates with increased uptake and activity of gemcitabine, while relative underexpression of hENT1 along the cell surface correlates with gemcitabine resistance[61].

Several studies have sought to test the prognostic potential of hENT1 receptor status in the clinical setting. One study performed immunohistological staining on tumor blocks of gemcitabine-treated pancreatic cancer[62]. These authors noted that in patients with detectable hENT1 protein staining in pancreatic tumor cells, median survival was significantly longer (13 mo *vs* 4 mo, $P = 0.01$) than in those patients with less abundant, heterogenous hENT1 staining. Another investigation identified hENT1 protein expression as highly correlative with clinical outcomes of disease-free survival, overall survival, and time to disease progression. Of concern with both these investigations, however, was the heterogenous patient population, including patients with both localized as well as advanced (metastatic) disease. Additionally, as both studies were retrospective analyses, it is difficult to make claims beyond those of a correlative nature.

Farrell *et al*[63] performed immunohistological staining for hENT1 on a tissue microarray of resected pancreatic tumors as part of the RTOG9704 study. These authors noted that hENT1 receptor expression was predictive of disease-free and overall survival in resected pancreatic cancer for those patients treated with gemcitabine but not 5-FU. Similarly, Kim *et al*[64] also found associations between low expressions of hENT1 protein and worse overall and disease-free survival in patients with resected pancreatic adenocarcinoma independent of gemcitabine therapy. However, no studies to date have investigated hENT1 expression in a neoadjuvant setting. As such, evaluation of hENT1 receptor status at the time of initial pancreatic tumor biopsy may prove advantageous in predicting eventual pathological and clinical responsiveness to gemcitabine-based chemotherapy.

Thymidylate synthase and dihydropyrimidine dehydrogenase

Thymidylate synthase (TS) is the intracellular enzyme responsible for synthesizing thymidine, a pyrimidine nucleoside required for DNA replication. 5-FU acts as a suicide inhibitor by irreversibly binding and inhibiting TS and thus preventing the production of deoxythymine monophosphate (dTMP)[65]. Without sufficient levels of dTMP, rapidly dividing cells are unable to synthesize DNA and therefore undergo apoptosis. Acting as a pyrimidine analogue, 5-FU is metabolized inside the cell into one of several possible cytotoxic metabolites which are incorporated into the actively synthesizing strands of DNA and RNA.

Both from an efficacy and a toxicity perspective, significant variability exists between patients treated with 5-FU. Such therapeutic unpredictability in response to 5-FU has been linked to the rate-limiting enzyme in 5-FU's metabolic pathway, known as dihydropyrimidine dehydrogenase (DPD)[66]. Interestingly, an estimated one

in ten individuals carries a genetic mutation rendering them unable to metabolize 5-FU to its active metabolite. Laboratory testing for this mutation is available and could be used to identify patients in whom 5-FU may be ineffective (or even toxic). While both TS and DPD have been shown to be upregulated in the setting of pancreatic cancer[67], genetic variations do exist. For example, one Japanese study identified that over half of Japanese pancreatic tissue samples expressed low levels of TS in combination with high levels of DPD[68]. Perhaps not surprisingly, another study from Japan concluded that high DPD mRNA levels within pancreatic tumor sections were associated with high rates of therapeutic response to S-1[69].

Several studies have investigated TS enzyme expression in pancreatic tumors as a prognostic variable. One study reviewed tissue cores from a retrospective series of 132 resected patients[70]. On immunohistological analysis, roughly two-thirds of patients had high intratumoral TS protein expression while the remaining one-third had low expression. The median survival of patients with low TS expression was longer than those with high TS expression, and high TS expression was identified as an independent predictor of mortality on multivariate analysis. Moreover, in the subset of patients who received adjuvant 5-FU, there was a significant survival advantage in patients with high TS protein expression. In contrast, adjuvant 5-FU did not influence survival in patients with low TS expression. From these data, the authors concluded that high TS expression is a poor prognostic marker in patients with resected pancreatic cancer, however these patients do benefit from adjuvant 5-FU therapy. Alternatively, a similar study investigating TS protein expression found conflicting results[71]. Again, TS expression was evaluated *via* immunohistochemistry in 98 patients following an R0 resection of pancreatic head cancer. These authors noted only 26% of these tumors demonstrated high TS expression, and, in contrast to the prior study, these authors concluded that TS predicted favorable disease-free, cancer-specific, and overall survivals. While the specific prognostic value of TS remains debatable, it is clear that genetic variations in the protein expression of TS and DPD may contribute to variable efficacy of 5-FU-based regimens.

Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is a transmembrane receptor responsible for a wide array of downstream signaling pathways involved in both normal cells and those undergoing carcinogenesis[72]. Pancreatic cancer cells are known to overexpress EGFR, and studies have demonstrated correlations between receptor/ligand coexpression and larger tumors, advanced clinical staging, and decreased survival[73,74]. As such, tyrosine kinase inhibitors are a novel class of therapeutic agents developed to act at one of the active binding sites along the receptor to prevent EGFR-mediated cell-cycle progression and cellular proliferation. Erlotinib is one of the most thoroughly investigated agents in the pre-clinical setting,

and this tyrosine kinase inhibitor has been shown to act synergistically with gemcitabine to exhibit extended antitumor activity in both *in vitro* and *in vivo* models[75-77].

Additionally, erlotinib has been a component of combination therapy. Unfortunately, despite reports of favorable safety and toxicity profiles, few studies have yielded breakthrough improvements in patient survival. Combination therapy of erlotinib and bevacizumab, a monoclonal antibody to vascular endothelial growth factor receptor (VEGF-R), found relatively little improvement in patients with advanced pancreatic cancer who failed previous gemcitabine therapy[78]. However, another recent phase III trial randomly assigned patients with unresectable locally advanced or metastatic pancreatic cancer to receive either gemcitabine or gemcitabine plus erlotinib[79]. One-year survival and disease-free survival were statistically significantly improved in those patients with combination therapy. Overall survival was statistically improved as well, though only by several weeks, which calls into question its clinical significance. EGFR inhibition has also been shown to act synergistically with chemoradiation in promoting antitumor properties[77].

Carbohydrate antigen 19-9

Carbohydrate antigen 19-9 (CA 19-9) is the most familiar cell-surface protein used in the management of pancreatic cancer[80,81]. It was first discovered in the serum of pancreatic and colon cancer patients in 1981 and has since been identified in other malignant and benign pathologies within the gastrointestinal tract[82]. Initial studies proposed CA 19-9 as a screening tool, but its relatively low sensitivity and specificity prevented its widespread adoption as a screening tool for pancreatic adenocarcinoma[83,84]. Traditionally, the utility of this overexpressed cellular surface protein has been in the assessment of response to chemotherapy and identification of tumor recurrence following resection, but data suggest CA 19-9 may also have a prognostic role[81,85]. A recent study of 324 patients with resectable pancreatic cancer correlated outcomes with various tumor markers, and the investigators demonstrated that a high preoperative CA 19-9 × carcinoembryonic antigen (CEA) index was an independent predictor of survival and strongly correlated with early postoperative mortality[86].

Two recent studies from MD Anderson evaluated CA 19-9 in patients who underwent neoadjuvant therapy prior to surgical resection. The first evaluated the relationship between CA 19-9 and surgical outcomes in patients with borderline resectable disease[87]. Normalization of CA 19-9 following neoadjuvant therapy was associated with longer overall survival in both resected (38 mo *vs* 26 mo; $P = 0.020$) and unresected (15 mo *vs* 11 mo; $P = 0.022$) patients. Conversely, failure of CA 19-9 to normalize was identified as an independent factor associated with shorter overall survival (HR = 2.13, $P = 0.001$). The second study evaluated the ability of CA 19-9 to predict completion of multimodality therapy involving neoadjuvant chemoradiation and surgical resection[88]. Although a low pretreatment CA 19-9 had a high positive predictive

value of completing neoadjuvant multimodality therapy, it concurrently demonstrated a low negative predictive value. Additionally, the investigators in this study found no association between a drop in CA 19-9 and histopathologic response to neoadjuvant multimodality therapy. From this, the authors discarded the notion of incorporating pretreatment CA 19-9 levels into their decision-making algorithm.

Secreted protein acidic and rich in cysteine

Beyond cell surface proteins and secreted molecules, increasing preclinical evidence is demonstrating the role of tumor microenvironments in the initiation, migration, basement membrane invasion, angiogenesis, and potential metastasis of pancreatic cancer[58]. One protein found in this microenvironment, the cell-surface molecule secreted protein acidic and rich in cysteine, or SPARC, is one of the most heavily researched stromal proteins[89]. Healthy pancreatic tissue typically stains faintly positive for the SPARC protein within acinar cells, islet cells, and fibroblasts within the pancreatic extracellular matrix. While normal ductal cells rarely stain positive, Guweidhi *et al*[90] noted a 31-fold increase in SPARC protein staining within pancreatic adenocarcinoma compared to normal tissue. (Interestingly, they also noted a 16-fold increase in SPARC protein expression in chronic pancreatitis.) One study identified positive immunohistological SPARC staining in 84% of retrospectively reviewed pancreatic adenocarcinoma tissue samples[91]. SPARC mRNA overexpression has been associated with both disease progression and poor prognosis in resected pancreatic tumors[92,93].

A recent phase III multi-institutional study published demonstrated a significant survival benefit in patients with stage-IV pancreatic cancer who received nab-paxlitaxel in combination with gemcitabine *vs* gemcitabine monotherapy[12]. Nab-paclitaxel's antitumor effects are found in the disruption of microtubule formation and disassembly during cellular mitosis, and this particular formulation exploits paclitaxel bound to albumin. This structure not only improves the side effect profile but also favors accumulation within tumor cells by the binding of albumin to SPARC. By binding to SPARC within the extracellular matrix, nab-paclitaxel successfully disrupts the organization of the tumor cells and induces a marked alteration in the tumor architecture, resulting in increased tumor softening and permeability[94]. These findings could prove beneficial from both chemotherapeutic delivery and surgical resection perspectives.

SMAD4

Another stromal-based protein gaining popularity in pancreatic cancer is SMAD4, a member of the Smad family. SMAD4 activity is muted in pancreatic cancer, and its specificity for pancreatic cancer makes it one of the most heavily investigated tumor markers[95]. This family of proteins signals through the transforming growth factor-beta (TGF-β) receptor, a major receptor in the pathogenesis of pancreatic cancer[96]. This transcription factor pathway

typically regulates cellular proliferation, differentiation, and apoptosis, and has been shown to act in tumor suppression, as well as tumor initiation and progression depending on the stage of carcinogenesis as well as cell type[97]. SMAD4 has also been implicated in promoting tumor metastasis in pancreatic cancer[98].

SMAD4 has been shown to be mutated in up to 50% of all pancreatic adenocarcinomas. Despite its clear association with the diagnosis, its prognostic role remains less distinct. Though some investigations suggest functional SMAD4 loss predicts a poor prognosis, other studies failed to demonstrate a relationship between SMAD4 mRNA expression and patient survival[99]. Due to the complexity of TGF-β signaling in pancreatic cancer, further investigations are needed to identify potential novel targeted therapies involving SMAD4.

Non-coding RNA

Beyond the classical protein products discussed previously, the field of epigenetics has begun to play an increasingly important role in the identification oncologic tumor markers and treatment optimization[100]. Unlike typical RNA molecules which code for functional proteins, non-coding RNA molecules themselves function in various methods to influence transcriptional and post-transcriptional regulation of gene expression[101]. In particular, one recent study suggested that the microenvironment of a tumor may stimulate a microRNA gene family that induces tumor resistance to therapy and promotes tumor cell invasion and metastasis[102]. Individual microRNA molecules are proving to have important treatment and prognostic value, as is found in a non-coding RNA named HOTAIR, which is upregulated in pancreatic tissue and has demonstrated pro-oncogenic function and an association with more aggressive tumor biology[103]. Preis *et al*[104] identified a consistent and significant overexpression of a microRNA named miR-10b in pancreatic cancer cells compared to benign tissue, while decreased expression of miR-10b was correlated with improved response to multimodality neoadjuvant therapy, likelihood of resection, delayed time to metastasis, and increased rate of survival. The field of research in epigenetics will likely support further studies on the identification of biomarkers diagnostic and therapeutic for pancreatic cancer.

While these select few biomarkers have been presented individually, it is likely they will prove most prognostic and therapeutically efficacious when analyzed as biomarker arrays instead of individual proteins. Indeed, patients harboring multiple mismatch repair gene polymorphisms have been associated with significantly worse survival compared to those patients with fewer (or no) mutations[105]. In the near future, gene expression analyses will likely play a significant role in the management of cancer patients, allowing for accurate prognostic information gleaned from the tissue at the time of initial biopsy.

CONCLUSION

In summary, patients with pancreatic adenocarcinoma

who undergo surgical resection coupled with chemotherapy and/or chemoradiation have the best opportunity for long-term survival. However, the multitude and variety of chemotherapeutic options demonstrate that no current regimen in our armamentarium is clearly superior to others. Variations in tumor biology, the presence or absence of molecular markers, a patient's functional status, and tolerability of potential side effects of current chemotherapeutic and radiation regimens make a simple and single universal therapeutic treatment modality difficult to advocate. In the meantime, there are a number of reasons to believe a neoadjuvant approach may be the best available strategy at this time, capitalizing on the critical concept of patient selection. Furthermore, molecular biomarkers such as hENT1, SPARC, and SMAD4 have gained recent popularity for their apparent predictive and prognostic abilities, and both epigenetic profiling and the identification of various oncologic microRNA molecules are likely to contribute to the field of pancreatic cancer treatment.

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2014 ADVANCES IN PANCREATIC CANCER

Utility of PET/CT in diagnosis, staging, assessment of resectability and metabolic response of pancreatic cancer

Xiao-Yi Wang, Feng Yang, Chen Jin, De-Liang Fu

Xiao-Yi Wang, Feng Yang, Chen Jin, De-Liang Fu, Pancreatic Disease Institute, Department of Pancreatic Surgery, Huashan Hospital, Fudan University, Shanghai 200040, China

Xiao-Yi Wang, Feng Yang, Chen Jin, De-Liang Fu, Department of Surgery, Shanghai Medical College, Fudan University, Shanghai 200032, China

Author contributions: Wang XY contributed to the conception and design of the review, data acquisition, analysis and interpretation, drafting and revising the article, and final approval of the version to be published; Yang F contributed to the conception and design of the review, revising it critically for important intellectual content and the final approval of the version to be published; Jin C and Fu DL contributed to the conception and design of the review and final approval of it.

Correspondence to: Feng Yang, MD, Pancreatic Disease Institute, Department of Pancreatic Surgery, Huashan Hospital, Shanghai Medical College, Fudan University, 12 Central Urumqi Road, Shanghai 200040, China. yffudan98@126.com
Telephone: +86-21-52887164 Fax: +86-21-52888277

Abstract

Pancreatic cancer is one of the most common gastrointestinal tumors, with its incidence staying at a high level in both the United States and China. However, the overall 5-year survival rate of pancreatic cancer is still extremely low. Surgery remains the only potential chance for long-term survival. Early diagnosis and precise staging are crucial to make proper clinical decision for surgery candidates. Despite advances in diagnostic technology such as computed tomography (CT) and endoscopic ultrasound, diagnosis, staging and monitoring of the metabolic response remain a challenge for this devastating disease. Positron emission tomography/CT (PET/CT), a relatively novel modality, combines metabolic detection with anatomic information. It has been widely used in oncology and achieves good results in breast cancer, lung cancer and lymphoma. Its utilization in pancreatic cancer has also been widely accepted. However, the value of PET/CT in pancreatic disease is still controversial. Will PET/CT change the treatment strategy for potential surgery candidates? What kind of patients benefits most from this exam? In this review, we focus on the utility of PET/CT in diagnosis, staging, and assessment of resectability of pancreatic cancer. In addition, its ability to monitor metabolic response and recurrence after treatment will be emphasis of discussion. We hope to provide answers to the questions above, which clinicians care most about.

Key words: Position emission tomography/computed tomography; Pancreatic cancer; Diagnosis; Staging; Metabolic response

Core tip: Position emission tomography/computed tomography (PET/CT) is a useful modality in the detection of pancreatic cancer, while its use in staging is limited by the lack of enhanced CT scan and a relatively poor sensitivity in detecting metastatic lymph nodes. It has the advantage in monitoring metabolic response, making it optimal in evaluation of different kinds of treatment and also in detecting suspected recurrence. The correlation between Standardized Uptake Value and prognosis remains controversial. Many efforts have been made to improve the diagnostic efficacy of PET/CT.

Original sources: Wang XY, Yang F, Jin C, Fu DL. Utility of

INTRODUCTION

Pancreatic cancer, one of the most common gastrointestinal tumors, remains a great threat to public health. In the United States, the estimated incidence of pancreatic cancer in 2013 ranks 10th for men and 9th for women. However, the estimated mortality ranked 4th for both sexes[1]. In China, from 1998 to 2007, the annual incidence for men and women showed an increase in both urban and rural area[2]. In 2009, pancreatic cancer incidence ranked 7th among all malignancies, with reported mortality ranking 6th[3]. The overall 5-year survival rate of pancreatic cancer is still extremely low, lesser than 5%[4,5]. Although surgery is a potential therapeutic method for long-term survival, the 5-year survival rate after radical resection fluctuates around 10%-29%[6-8].

To date, standard diagnostic workup for pancreatic cancer includes conventional imaging such as multi-detector computed tomography (MDCT), magnetic resonance imaging (MRI), endoscopic ultrasound (EUS), as well as invasive procedures such as EUS-guided fine-needle aspiration (EUS-FNA). MDCT remains the most widely used imaging modality for cancer staging. It makes the golden standard for local infiltration. However, missing of small liver metastasis has been reported[9]. Although MRI has been widely used for evaluation of pancreatic lesions, its overall value is controversial[10]. Recently, EUS has been more widely used in detection of clinically suspected pancreatic lesions. With FNA, it has been reported to be the most accurate imaging technique for pancreatic neoplasms[11,12]. However, Doppler ultrasonography including contrast enhancement also has limitations, such as blooming artifacts, poor spatial resolution, and low sensitivity (SE) to slow flow[13-15].

Increased glycolysis is a characteristic metabolic feature of malignant tumors[16]. Although many tracers have been introduced, 18F-fluorodeoxyglucose (18F-FDG), which aims to glucose metabolism, remains the most widely used one. After converted into 18FDG-6-PO4, it does not continue along the glycolytic cycle and accumulates in cancer cells. Based on this principle, positron emotion tomography (PET) was introduced in 1976. However, the lack of precise anatomic information had limited its use. Since the combination of PET and CT in 1999[17], PET/CT had been widely applied in oncology. In this review, we focus on the utility of PET/CT in the diagnosis, staging, and assessment of resectability and metabolic response of pancreatic cancer.

PET/CT IN DIAGNOSIS OF PANCREATIC CANCER

PET has always been reported to be a highly sensitive and accurate method for detecting pancreatic cancer. The reported SE ranges from 78% to 95%, and accuracy from 64% to 91%[18-25]. The combination of PET and CT improves them to 85%-97%, and 85%-95%[26-32]. However, the specificity (SP) is relatively low and varies greatly among different studies, with 50%-87% for PET alone[18-25] and 61%-94% for PET/CT[26-32]. Several studies on utilization of PET/CT in diagnosis of pancreatic cancer are shown in Table 1. A meta-analysis conducted by Tang *et al*[33] showed a pooled SE of 90.1%, with an SP of 80.1%. Another meta-analysis by Wu *et al*[34] revealed similar results with a pooled SE of 87% and an SP of 83%. The possible reason for the relatively low SP may be misdiagnosis of mass forming pancreatitis as tumors on PET imaging.

The differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma has always been a challenge. Long-term chronic inflammation will lead to rich fibrosis of pancreatic parenchyma which makes the lesion appear as a low density mass on CT with a weak or no enhancement[19]. The reported SE and SP of CT for differentiating chronic pancreatitis from cancer were 82%-94% and 83%-90%, respectively[35]. MRI showed similar results as CT, with the SE and SP of 93% and 87%, respectively[36].

18FDG-PET was once thought to be the solution to this problem. Reske *et al*[37] reported that the overexpression of glucose transporter 1 was generally increased in pancreatic cancer but not in chronic pancreatitis, which revealed the possibility of diagnosing pancreatic cancer from mass-forming pancreatitis. Positive results were reached by Imdahl *et al*[38] in 1998 and by van Kouwen *et al*[19] in 2004 through prospective study. Detailed information of PET/CT in differential diagnosis of pancreatic carcinoma and mass-forming pancreatitis is showed in Table 2. However, value of FDG-PET/CT in differential diagnosis of pancreatic cancer from chronic pancreatitis is still controversial, as a consensus has not been reached on whether or when PET/CT should be applied.

FDG uptake caused by increased glycolytic activity has been shown in inflammatory cells such as neutrophils and activated macrophages[39,40]. Accordingly, FDG has been reported to accumulate in various inflammatory processes, including acute pancreatitis[41], auto-immune pancreatitis[42-45], tuberculosis[46,47], and mass-forming chronic pancreatitis. High 18FDG-uptake by mass forming chronic pancreatitis has been also reported by many studies[27,48,49]. A recent study by Kato *et al*[50] indicated that differentiation between metastasis-free pancreatic cancer and mass-forming pancreatitis was difficult by FDG-PET/CT due to considerable overlapping between

Table 1 Position emission tomography/computed tomography in detection of malignant pancreatic tumors

Ref.	Study design	Maligancy/all (*n*)	SUV (max) of malignant lesions (mean ± SD)	SUV (max) of benign lesions (mean ± SD)	Cutoff value	SE	SP	PPV	NPV	LR(+)	LR(-)	Accuracy
Keogan *et al*[24]	R	25/37	5.4	1.4	-	88.00%	83.33%	91.67%	76.92%	5.28	0.144	86.49%
[1]Rose *et al*[23]	R	52/65	5.0 ± 1.2	0.85 ± 0.1	-	92.30%	84.62%	96.00%	73.33%	6	0.09	90.76%
[1]Delbeke *et al*[22]	R	52/65	5.1 ± 2.6	0.85 ± 1.7	3.0	92.30%	84.62%	96.00%	73.33%	6	0.09	90.76%
[2]Lemke *et al*[20]	R	64/100	-	-	3.5	84.37%	61.11%	79.41%	68.75%	2.17	0.26	76.00%
[1]Lytras *et al*[18]	R	72/112	-	-	-[3]	73.00%	60.00%	80.00%	49.00%	-	-	64.00%
Heinrich *et al*[32]	P	46/59	-	-	-	89.13%	69.23%	91.11%	64.29%	2.89	0.16	84.75%
Nishiyama *et al*[31]	R	55/86	5.75 ± 2.69	3.69 ± 1.58	3.5	89.09%	70.97%	84.48%	78.57%	3.07	0.15	82.56%
Bang *et al*[30]	R	93/102	5.1 ± 2.1	3.2 ± 1.8	-	96.77%	77.78%	97.82%	70.00%	4.35	0.04	95.09%
Kauhanen *et al*[29]	P	19/38	4.85 ± 2.77	2.25 ± 0.75	2.6	85.00%	94.44%	94.44%	85.00%	15.3	0.16	89.47%
Buchs *et al*[28]	R	36/45	6.5 ± 4.5	3.4 ± 3.1	-	72.00%	33.30%	80.00%	25.00%	-	-	64.00%
[4]Buchs *et al*[28]	R	36/45	6.5 ± 4.5	3.4 ± 3.1	-	96.00%	66.60%	92.30%	80.00%	-	-	90.30%
Santhosh *et al*[27]	R	57/87	8.64 ± 5.21	4.86 ± 4.54	2.8	96.36%	78.57%	94.64%	84.61%	4.49	0.05	92.75%
Hu *et al*[26]	R	54/80	6.3 ± 2.4	2.9 ± 2.0	3.5	96.29%	72.72%	89.65%	88.89%	3.53	0.05	89.47%

[1]Fluorodeoxyglucose-position emission tomography (FDG-PET) scan without computed tomography (CT); [2]Voxel-based retrospective registration and fusion of CT and PET were performed with software. PET imaging and CT were not taken at the same time; [3]Lesions measured visually; [4]Data obtained with extra scan of enhanced PET/CT. SE: Sensitivity; SP: Specificity; NPV: Negative predictive value; PPV: Positive predictive value; R: Retrospective study; P: Prospective study.

Table 2 Position emission tomography/computed tomography in differential diagnosis of pancreatic carcinoma and mass-forming pancreatitis

Ref.[1]	Study design	PC/CP	SUV(max) of PC (mean ± SD)	SUV(max) of CP (mean ± SD)	Cutoff value	SE	SP	PPV	NPV	LR(+)	LR(-)	Accuracy
Stollfuss *et al*[25]	R	43/30	3.16 ± 1.22	1.00 ± 0.55	1.53	93.18%	93.10%	95.35%	90.00%	13.51	0.07	93.15%
Mertz *et al*[21]	R	31/4	-	-	2.80	87.09%	50.00%	93.33%	33.33%	1.74	0.25	82.86%
van Kouwen *et al*[19]	R	32/77	-	-	-[2]	90.62%	87.01%	74.35%	95.71%	6.97	0.11	88.07%
Lytras *et al*[18]	R	54/25	-	-	-[3]	78.00%	55.00%	78.00%	55.00%	-	-	64.00%

[1]Fluorodeoxyglucose-position emission tomography (FDG-PET) scan without computed tomography (CT); [2]Results were judged to be abnormal if focal accumulation of the tracer was detected in the area of the pancreas. Faint and/or diffuse FDG uptake in the pancreatic region (*i.e.*, uptake slightly higher than the surrounding background, but clearly lower than the liver) was not considered suspicious for pancreatic cancer; [3]Lesions measured visually. SE: Sensitivity; SP: Specificity; NPV: Negative predictive value; PPV: Positive predictive value; R: Retrospective study; P: Prospective study.

the Standardized Uptake Value (SUVmax) values of these two diseases.

Dual-phase [18]FDG imaging has been supposed to improve diagnostic efficacy. Mean value of SUVdelayed was significantly higher than that of SUVearly ($P < 0.01$) in pancreatic cancer. In benign pancreatic disease, there was a tendency of decreased SUVdelayed compared to SUVearly, but there was no significant difference in the mean values. Retention index [RI = (SUVdelayed-SUVearly) × 100/SUVearly] had a diagnostic accuracy of 88% and an SE of 93% for suspected pancreatic cancer[31]. Recent studies[50] revealed that the ranges of SUV(max) for pancreatic cancer and mass forming pancreatitis were mostly overlapped.

[18]FDG with enhanced CT was another attempt to improve diagnostic efficacy. In the study by Buchs *et al*[28], the statistical parameters of enhanced PET/CT surpassed those of unenhanced one, although none of them was of statistical significance (SE: 96% *vs* 72%, $P = 0.076$; SP: 66.6% *vs* 33.3%, $P = 0.52$; accuracy 90.3% *vs* 64%, $P = 0.085$).

PET/CT IN STAGING AND ASSESSMENT OF RESECTABILITY OF PANCREATIC CANCER

Precise pre-operative staging is crucial to make appropriate treatment decisions. Generally, resectability of pancreatic cancer concerns two problems: local tumor invasion of major vascular structures and distant metastasis. The ultimate goal is to save patient from unnecessary surgical exploration.

In most medical centers, an enhanced CT scan is not included in the routine PET scan. The plain CT is used for location only, thus limiting PET/CT's value in T staging. Wakabayashi *et al*[51] reported that FDG-PET without enhancement only detected 22.2% (2/9) of cases of invasion into the major arteries while CT found all 9 cases (100%). Strobel *et al*[52] reported using contrast-enhanced ^{18}F-FDG PET/CT to detect all five arterial infiltrations (100%/100%). However, PET and unenhanced PET/CT failed to detect arterial infiltration in all 5 cases

Table 3 ^{18}F-fluorodeoxyglucose-position emission tomography/computed tomography in N-staging and detection of liver metastasis of pancreatic cancer

Ref.	Study design	SE (%) (true positive/total positive)		
		PET/CT	CT	*P* value
N-staging				
Heinrich *et al*[32]	P	21.42 (3/14)	-	-
Maemura *et al*[59]	R	50.00 (3/6)	66.67 (4/6)	0.56
[1]Wakabayashi *et al*[51]	P	57.10 (8/14)	78.6 (11/14)	0.42
Kauhanen *et al*[29]	P	38	-	-
[1]Imai *et al*[60]	R	0 (0/6)	0 (0/6)	
Detection of liver metastasis				
Fröhlich *et al*[63]	R	68 (15/22)		
Mertz *et al*[21]	R	78 (7/9)	33.33 (3/9)	0.06
Lytras *et al*[18]	R	22	20	0.81
Heinrich *et al*[32]	P	81 (13/16)	56 (9/16)	0.22
Maemura *et al*[59]	R	37.5 (3/8)	87.5 (7/8)	0.04
Wakabayashi *et al*[51]	P	52.6 (10/19)	73.7 (14/19)	0.18
Farma *et al*[62]	R	61	57	
Strobel *et al*[52]	R	46 (5/11)		
Kauhanen *et al*[29]	P	88(6/7)	42.86 (3/7)	0.09

[1][18]F-fluorodeoxyglucose-position emission tomography (FDG-PET) scan without computed tomography (CT). SE: Sensitivity; SP: Specificity; NPV: Negative predictive value; PPV: Positive predictive value; R: Retrospective study; P: Prospective study.

(0%/100%).

Pancreatic carcinoma tends to transfer to lymph nodes at an early stage. In a study by the Japanese Pancreas Society (JPS), 306 of 822 TS1 (tumors < 2 cm in diameter) pancreatic cancer (37.2%) already had lymph node metastasis[53]. Kat'uchová *et al*[54] also reported that out of 319 histopathologically negative lymph nodes (34 patients), 134 lymph nodes were classified as immunohistochemically positive (21 patients). The detection of metastatic lymph nodes has always been a challenge. CT can only detect lymphadenopathy which may also be caused by inflammation. Lymph node size is not a reliable parameter for the evaluation of metastatic involvement[55]. FDG-PET/CT has reached good results in the N staging of non-small cell lung cancer, periorbital malignancies and nasopharyngeal carcinoma[56-58]. However, its utilization in pancreatic cancer is limited. The reported SE of FDG-PET/CT for detecting metastatic lymph nodes ranges from 21%-38%[20,29,32]. Maemura *et al*[59] reported an SE of 50% for para-aortic lymph node, while Imai *et al*[60] reported an SE of 0%. Detailed information is showed in Table 3. Lesions that smaller than 5 mm in diameter are hard to detect even for FDG-PET/CT. The low metabolic state and partial volume effect may be the reasons. Thus, it is improper to decide the necessity and range of lymphadenectomy based on FDG-PET/CT pre-operative N-staging results.

As a whole body exam, PET/CT possesses the unparalleled advantage in M staging. The reported SP is as high as 91%-100%. Strobel *et al*[52] reported an SE of 100% for detecting lung and bone metastases. Kitajima *et al*[61] reported three pancreatic cancer patients with ovarian metastases detected only by FDG-PET/CT. In the study by Strobel *et al*[52], unenhanced and enhanced PET/CT had accuracies of 60% and 80% for detecting peritoneal implantation. Farma *et al*[62] also reported two peritoneal metastases found by PET/CT alone. The particular SE for detecting liver metastasis, however, dropped to 22% to 88%[18,21,29,32,51,59,62,63]. The detailed information of studies focused on the detection of liver metastasis by FDG-PET/CT is showed in Table 3. One of the possible reasons may be that the detection of small liver metastatic lesions is limited by partial volume effects[64]. The high metabolic background of the liver may be another reason[56].

The overall influence of ^{18}F-FDG PET/CT on the management of pancreatic cancer has been widely studied. In early years, FDG-PET without CT did not perform well. Wakabayashi *et al*[51] reported that FDG-PET only surpassed CT in the detection of bone metastasis and concluded that PET did not perform precisely enough in staging of the disease. Since then, many studies revealed the capability of FDG-PET/CT to evaluate pre-operative staging by providing extra information. In the study conducted by Farma *et al*[62], 11% (7/82) of patients with invasive cancer had a change in their management, as PET/CT detected metastatic lesions that were not identified by the standard staging protocol in these patients. Bang *et al*[30] reported that [18]FDG-PET/CT changed the pretreatment stage in 26.9% (25/93) of patients with pancreatic ductal adenocarcinoma. More importantly, [18]FDG-PET/CT scanning resulted in a change in resectability status in 20 cases (21.5%). Although some investigators hold a negative opinion[29], PET/CT plays a critical role in changes in the management of pancreatic cancer[21,59,65,66].

PET/CT IN TUMOR RECURRENCE DETECTION AND METABOLIC RESPONSE MONITORING

Early detection of tumor recurrence and accurate postoperative staging are crucial for prescribing optimal individualized treatment[67,68]. Elevation of serum level of CA19-9 has been shown to be a sensitive indicator of recurrent pancreatic cancer but did not provide information about location of recurrence[69]. For patients who underwent surgery, PET/CT is able to detect recurrence early during the follow-up. Ruf *et al*[70] conducted a study including 31 patients with suspected recurrence after surgery. Among the 23 patients with local recurrence, the detection rate of FDG-PET was 96%, while that of CT/MRI was 39%. Among 12 liver metastases, the detection rate of FDG-PET was 42%, while that of CT/MRI was 92%. Other malignant abdominal lesions were detected by FDG-PET only. Similar results were reported by Sperti *et al*[71]. In their study, tumors recurred in 63 of 72 (87.5%) patients. Tumor relapse was detected by CT in 35 patients, while by FDG-PET in 61. FDG-PET influenced treatment strategies in 32 of 72 patients

(44.4%). The confirmation of recurrent pancreatic cancer in the remnant pancreas has also been reported by other researchers[72,73].

FDG-PET/CT's ability to detect the metabolic change before morphological changes has been proven by *in vivo* studies[74,75]. It has been successfully utilized in monitoring the metabolic changes during chemotherapy and/or radiation therapy. Chang *et al*[76] reported that PET-CT was a more effective method for evaluating tumor response than conventional CT after radiotherapy for unresectable pancreatic cancer. In another study[77], CT and FDG-PET were done before and after arterial infusion chemotherapy combined with external radiation therapy (ERT) for unresectable patients. CT could not reveal the actual location of the tumor before treatment in two cases. PET image showed high uptake in the pancreatic head before treatment and the significant decrease of SUV after treatment. In addition, FDG-PET image showed therapeutic effects 2 mo before changes appeared on CT images in another two cases. Heinrich *et al*[78] reported a significant SUV decrease (mean SUV from 4.4 to 3.0) that occurred during chemotherapy (*P* = 0.031) for locally advanced pancreatic cancer (LAPC). Their results were confirmed by many other studies[30,79-82]. With a wide approval in monitoring metabolic response, PET/CT now engages in clinical trials on novel drugs such as nab-paclitaxel[83].

PET/CT IN PREDICTION OF PROGNOSIS

Proliferation index is important for malignant potential in pancreatic cancer and neuroendocrine tumors (NETs). Buck *et al*[84] found that Ki-67 immunoreactivity enabled reliable differentiation between benign and malignant pancreatic tumors. The mean percentage of Ki-67 positive cells was approximately ten-fold higher in pancreatic cancer than in pancreatitis, indicating that proliferative activity is elevated strongly in the former but only slightly in the latter. However, no significant correlation was found between Ki-67 immunoreactivity and FDG uptake (*P* = 0.65). Their results accorded with *in vitro* results, which indicated no correlation between proliferative activity and FDG uptake in human cancer cells[85].

Whether [18]FDG PET is a prognostic factor for patients with pancreatic cancer is debatable. In a study by Sperti *et al*[86], SUV value of [18]FDG was calculated in 60 of the patients and divided into high (> 4) and low (≤ 4) groups. The median survival for patients with SUVs > 4.0 (*n* = 29) was 265 d *vs* 178 d for those with SUVs ≤ 4.0 (*n* = 31) (*P* = 0.005). Multivariate analysis showed that only stage (*P* = 0.001) and SUV (*P* = 0.0002) were independent predictors of survival. Similar results were obtained by Zimny *et al*[87] using a cutoff value of 6.1. Epelbaum *et al*[88] confirmed that global 18F-FDG influx (^{18}F-FDG INF) was the only significant variable for overall survival (OS) in patients with localized disease, independent of resectability.

Correlation between metabolic response on FDG-

PET and prognosis is still controversial. Results varied greatly among various studies. Topkan *et al*[89] conducted a study including 32 unresectable LAPC patients treated with concurrent chemoradiotherapy. Median OS, progression-free survival (PFS), and local-regional PFS for those with greater (*n* = 16) *vs* lesser (*n* = 16) SUV (max) change were 17.0 mo *vs* 9.8 mo (*P* = 0.001), 8.4 mo *vs* 3.8 mo (*P* = 0.005), and 12.3 mo *vs* 6.9 mo (*P* = 0.02), respectively. On multivariate analysis, SUV (max) difference was predictive of OS, PFS, and LRPFS, independent of existing covariates. The great SUV decrease indicating better prognosis was also confirmed by several other studies[60,78,88]. On the contrary, Heinrich *et al*[78] revealed that significant SUV decrease occurred during chemotherapy was correlated with Ki-67 expression (*P* = 0.016), and histologic response (*P* = 0.01), while the metabolic response was not predictive of the median disease-free survival (*P* = 0.49) or OS (*P* = 0.43).

NEW DEVELOPMENTS AND PROSPECTS

The fusion of PET and MRI has shown more accurate localization of the FDG uptake in relation to the pancreatic ductal system[89,90]. Tatsumi *et al*[91] showed that the diagnostic accuracy was higher on PET/T1-w or PET/T2-w MRI (93.0 and 90.7%, respectively) than PET/CT (88.4%), although no statistical significance was obtained. Nagamachi *et al*[92] showed that FDG-PET/MRI fusion image, which provided more anatomic information, significantly improved accuracy compared with PET/CT (96.6% *vs* 86.6%). Dilatation of main pancreatic ducts was noted in 65.9 % of solid types and in 22.6% of cystic types on PET/MRI-T2 fusion images. Especially in cystic types, intra-tumor structures such as mural nodules (35.4%) and intra-cystic septum (74.2%) were also detected.

With regard that pancreas is located at a relatively greater distance from the diaphragm, respiratory gating procedure does not ameliorate the diagnostic assessment of primary tumors. Furthermore it could be useful to improve staging both in the liver and lung. In default of respiratory gating equipment, Kasuya *et al*[93] suggested that deep-inspiration breath-hold PET/CT technique seems feasible for accurate localization and improves the quantification of SUV. Further investigation is needed about the real application of these new procedures and protocols.

The finding of more tumor specific tracers is another major endeavor. The most widely reported ^{18}F-FET assesses proportion of cells undergoing active proliferation. von Forstner *et al*[94] demonstrated FLT uptake in Panc-TuI and BxPC-3 pancreatic cancer cell lines. However, the outcomes of clinical studies were controversial[95,96]. The hypoxia agent ^{18}F-FMISO, aimed at the hypoxic environment of pancreatic cancer, was compared with FDG by Segard *et al*[97]. In their study, only 2 pancreatic cancer patients demonstrated increased FMISO activity, while all ten patients showed FDG uptake. Mean FDG SUV (max) was 6 (range: 3.8-9.5) compared to 2.3 for

FMISO (range: 1-3.4). Other reported tracers included choline analogues (^{11}C-CHO, ^{18}F-dOC)[98] and ^{11}C-harmine[99]. The most recent pilot study used antibody like anti-CD147 monoclonal antibody[100] as a probe or even targeted mutant KRAS2 mRNA with ^{111}In-DOTAn-Poly(diamidopropanoyl)m-KRAS2 PNA-D(Cys-Ser-Lys-Cys) nanoparticles[101]. However, none of them is able to replace FDG at the time being. Further study in this field is still needed. Another kind of novel tracers worth noticing is somatostatin receptor (SSTR) tracers, like Yttrium-labelled peptides[102], which are used for imaging and peptide receptor-mediated radiotherapy for pancreatic NETs. Around 80% of enteropancreatic NETs express SSTRs, with some differences in different tumor types and even within the same tumor[103]. Recently, Putzer *et al*[104] reported ^{68}Ga-DOTA-TOC PET imaging to be an established imaging procedure for accurate staging for NET patients. ^{68}Ga-DOTA-TOC revealed more tumor sites than ^{68}Ga-DOTA-LAN. The tumor to background ratios for tumor and liver calculated from SUV(max) measurements were significantly higher for ^{68}Ga-DOTA-TOC than ^{68}Ga-DOTA-LAN ($P < 0.02$).

In conclusion, FDG-PET/CT is a useful modality for detection of pancreatic cancer. Its false positive findings in mass forming pancreatitis may lower its specificity. Its use in tumor staging is limited by the lack of enhanced CT scan and a relatively poor SE in detecting metastatic lymph nodes. However, for most of the time extra information about distant metastasis is vital enough to change clinical management. FDG-PET/CT has the advantage in monitoring metabolic response, making it optimal in evaluation of different kinds of treatments. It is also a valuable tool to detect suspected recurrence. The correlation between SUV and prognosis remains controversial. Many efforts have been made to improve diagnostic efficacy of PET/CT. Though the outcome is not sufficient today, more possibility may lay in the future.

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2014 ADVANCES IN PANCREATIC CANCER

Central pancreatectomy: The Dagradi Serio Iacono operation. Evolution of a surgical technique from the pioneers to the robotic approach

Calogero Iacono, Andrea Ruzzenente, Luca Bortolasi, Alfredo Guglielmi

Calogero Iacono, Andrea Ruzzenente, Luca Bortolasi, Alfredo Guglielmi, Department of Surgery, Division of General Surgery "A", Unit of Hepato-Biliary-Pancreatic Surgery, University of Verona Medical School, "GB Rossi" University Hospital, 37134 Verona, Italy

Author contributions: Iacono C study concept and design; Bortolasi IC, Ruzzenente A acquired the data; Iacono C and Bortolasi L drafted of the manuscript; Iacono C, Guglielmi A and Ruzzenente A critical revision of the manuscript for important intellectual content; Iacono C and Guglielmi A study supervisor and final approval.

Correspondence to: Calogero Iacono, MD, Professor, Department of Surgery, Division of General Surgery "A", Unit of Hepato-Biliary-Pancreatic Surgery, University of Verona Medical School, "GB Rossi" University Hospital, P.le L.A. Scuro, 37134 Verona, Italy. calogero.iacono@univr.it

Telephone: +39-45-8074412   Fax: +39-45-8027426

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Abstract

Central pancreatectomy (CP) is a parenchyma-sparing surgical procedure. The aims are to clarify the history and the development of CP and to give credits to those from whom it came. Ehrhardt, in 1908, described segmental neck resection (SNR) followed, in 1910, by Finney without reconstructive part. In 1950 Honjyo described two cases of SNR combined with gastrectomy for gastric cancer infiltrating the neck of the pancreas. Guillemin and Bessot (1957) and Letton and Wilson (1959) dealt only with the reconstructive aspect of CP. Dagradi and Serio, in 1982, performed the first CP including the resective and reconstructive aspects. Subsequently Iacono has validated it with functional endocrine and exocrine tests and popularized it worldwide. In 2003, Baca and Bokan performed laparoscopic CP and, In 2004, Giulianotti *et al* performed a robotic assisted CP. CP is performed worldwide either by open surgery or by using minimally-invasive or robotic approaches. This confirms that the operation does not belong to whom introduced it but to everyone who carries out it; however credit must be given to those from whom it came.

Core tip: Central pancreatectomy (CP) is a parenchyma-sparing surgical procedure. Dagradi and Serio, in 1982, performed the first CP including the resective and reconstructive aspects. Before the description of Dagradi and Serio the previous descriptions of CP included only different parts of the complete surgical technique. Subsequently Iacono has validated it with functional endocrine and exocrine tests and popularised it worldwide. Nowadays CP is performed worldwide either by traditional open surgery or by minimally-invasive or robotic approaches.

INTRODUCTION

Since the 1980s, the panorama of pancreatic resections has widened, with the development of new operations

Figure 1 Dr. JMT Finney (courtesy of National Library of Medicine National Institutes of Health Department of Health and Human Services Bethesda, MD, United States).

other than proximal and distal pancreatectomy (DP). The aims of these operations have been to spare normal pancreatic parenchyma. These new operations are duodenum-sparing pancreas head resection[1], resection of ventral or uncinate process of the pancreas[2], dorsal pancreatectomy[3], middle preserving pancreatectomy[4] and central pancreatectomy (CP)[5].

Although CP has gained its place in the clinical armamentarium of surgeons worldwide, many authors have reported incorrect information regarding the history of this operation, and subsequent authors have perpetuated this misinformation, because they not have carefully read and considered the original papers and have simply repeated the same wrong information without verification. This has been confirmed in our systematic review and meta-analysis of the papers published in English literature[6,7].

The aim of this report was to clarify the history of this technique in an attempt to avoid the emergence and perpetuation of inaccuracies about the origin and development of the procedure.

Data sources
Reviewing all the papers for a systematic review and meta-analysis, published on *British Journal of Surgery* 2013; 100: 873-885[7], we found out that most of the authors reported wrong information or believed to have discovered the technique.

Credit for the first description of segmental pancreatic resection must be given to Oskar Ehrhardt of the Elisabeth-Krankenhaus in Konigsberg, Prussia who published in 1908 on Dtsch med Wochenschr[8]. Finney[9] describes the operation performed by Ehrhardt in 1907 as follows: "Woman age 32 years; three months previously a gastroenterostomy had been performed for cancer of the pylorus. Operation showed cancer of pylorus and a separate tumor in head of pancreas. Stomach and upper portion of duodenum together with most of head and body of pancreas resected including duct of Wirsung. The two extreme ends of pancreas were left. These were

then brought together by sutures and drained with gauze strips. Pancreatic fistula resulted. After two weeks piece of necrotic pancreas discharged through wound after which fistula closed". In the original paper in German, Ehrhardt described that at 4 mo from the operation the patient suffered of local unresectable tumor recurrence and died after 1 mo from the diagnosis of recurrence. The original description in German reporting two cases reads[8]: "Der erste Fall betraf eine 32 jährige Frau mit einem seit etwa einem halben Jahr bestehenden Pyloruscarcinom. Im Mai 1907 fand sich bei der Laparotomie ein überfaustgroßes Carcinom des Pylorus. im Pankreaskopf fühlte man einen zweiten Tumor [ich führte] die Gastroenterostomia retrocolica post. [durch]. Am 4. August 1907 machte ich die Relaparotomie... man musste zunächst den Magen resezieren [ich] musste die Resektion am Duodenum bis in den absteigenden Teil fortsetzen. Ich nahm noch große Stücke des Pankreas fort. In der Tiefe dieses Defekts war ein Gang, der Ductus Wirsungianus eröffnet. Ich beschloß die Wundränder im Pankreas aneinander zu nähen, und hatte die Freude, dass ich wenigstens die Gegend des Ductus Wirsungianus adaptieren und so eine Art von Naht des Ganges machen konnt Ich legte einen Vioformgazestreifen in die Pankreaswunde. Der zweite Wundverlauf war ein guter, doch blieb an der Tamponstelle eine Fistel, aus der sich nach zwei Wochen ein ziemlich großer Sequester des Pankreas ausstieß. Hiernach verheilte die Fistel.

Dieser güntige Zustand hielt indessen nur 4 Monate an, im Dezember kam Patientin miteinem großen,inoperabeln Rezidiv zumir. Es handelte sich um ein großß Drüsenrezidiv, an dem Patientin 5 Monate nach der Resektion zugrunde ging.

Im zweiten fall, den ich nur kurz schildern will, da hier die Resektion des Pankreas wenig ausgedehnt war, handelte es sich gleichfalls um ein Pyloruscarcinom. Auch hier war der Tumor mit dem Pankreaskopf verwachsen. [Ich] konnte ihn im Pankreas abtrennen ohne mit den Arterien oder dem Ausführungsgang in Collision zu kommen. Ich nähte die Drüsenkapsel. über dem Defekt zusammen und schloß die Bauchwunde ohne zu tamponieren. Ich habe von der Patientin noch 1 ½ Jahre nach der Operation günstige Nachrichten gehabt."

Finney[9] (Figure 1) in 1910 described segmental pancreatic neck resection for a cystic tumour. The two stumps were oversewn and not drained enterically and, as expected, the patient developed a pancreatic fistula. The reported description of the operation reads[9]. "The tumor would not shell out, and the whole middle portion of the gland was removed with it, leaving a small area each of the head and tail. These two fragments were brought together with mattress sutures of catgut. The patient bore the operation well. A fistula developed at the site of the drain which continued for about three months. Three times, including our own case, the gland has been completely divided and then the remaining portions brought together and sutured. All three cases recovered".

For his time, Finney, first president of the American
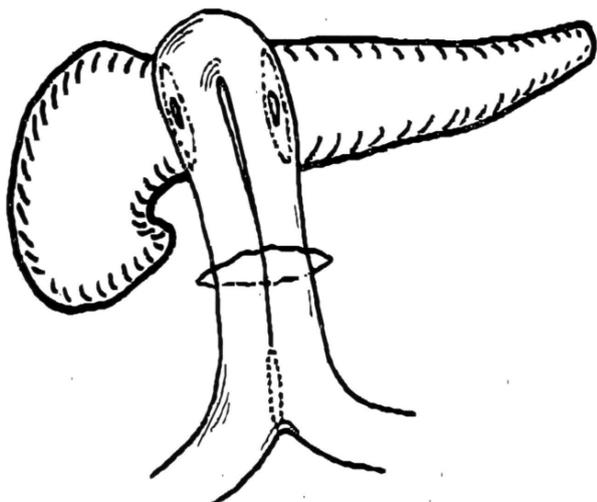
Figure 2 Technique performed by Guillemin and Bessot to reconstruct an accidental pancreatic transection while searching for the Wirsung duct for intraoperative wirsungraphy (published in *Mem Acad Chir Paris*; 83: 869-871, with permission of Mem Acad Chir Paris).

College of Surgeons[10], described a unique operation, however reconstruction was made by suturing together the two stumps. Suturing together two pancreatic stumps is not currently considered appropriate. Nonetheless it has been reported recently with anecdotal success in three patients (one with mucinous cystadenoma and two with traumatic transection of the pancreatic neck)[11] and in experimental mongrel dog model[12] with an end-to-end anastomosis between the two pancreatic stumps, and with pancreatic duct anastomosis (duct-to-duct) with or without stenting.

Takada *et al*[13] ascribed the first CP operation to the work done by Honjyo in 1950[14]. This paper which was published in Japanese, and unfortunately, for which translation has been not available until now in literature, described two cases of central resection of the pancreas combined with gastrectomy for gastric cancer infiltrating the neck of the pancreas. Although Honjyo did a central resection, the distal pancreatic stump was not reconstructed. Instead it was oversewn and wrapped with omentum in attempt to prevent pancreatic leakage. The proximal stump was also oversewn.

The primary closure of distal pancreatic stumps without entero-pancreatic anastomosis is currently not considered appropriate. Nonetheless primary closure of the distal stump was recently reported, surprisingly, without any pancreatic fistulation[15].

Guillemin and Bessot[16] in 1957 or Letton and Wilson[17] in 1959 suggested by some authors as first Authors to introduce CP, described only the reconstructive aspect of CP. In fact Guillemin and Bessot[16] only carried out transection of the isthmus followed by a double digestive anastomosis of the two pancreatic stumps to an omega-shaped jejunal loop (Figure 2).

Guillemin and Bessot[16] treated a patient with calcifying chronic pancreatitis and renal tuberculosis. In an attempt to visualize the main pancreatic duct, they inadvertently transected the entire pancreatic neck and decided to drain the two pancreatic stumps with an omega jejunal loop.

The original description of their operation (in French) reads. "Intervention le 27 mars 1957: le pancreas volumineux est induré, rigide, sur toute son étendue, On s'efforce de découvrir par punction le canal de Wirsung, puis de pratiquer une pancréatographie. On ne recuille pas de liquide, et les clichés ne son pas concluanis. l'incision du pancreas a la jonction de la tete avec l'isthme ne permet pas de trouver le Wirsung. On arrive ainsi sur le confluence portal. Devant cette situation, aprés avoir realize une sectione transversale complete du pancreas on prend la decision de faire une double anastomose du sommet de l'anse jéjunale avec chacune des tranches de section pancréatique. Anastomose pancréato-jéjunale en deux plans aux points séparés, solidarisation par quelques points des branches afférente et efferente de l'anse jéjunale qui est amarrée au mésocolon tranverse, anastomose latéro-latérale jéjuno-jéjunale au pied de l'anse[16]".

Letton and Wilson[17] performed the reconstructive aspect of CP by oversewing the cephalic stump with interrupted sutures and carrying out a pancreatico-jejunostomy to the distal stump. However this reconstruction was performed in a patient with traumatic transection of the neck of the pancreas, although no pancreatic parenchymal resection was performed (Figure 3).

In 1982, Dagradi (Figure 4) and Serio planned and performed the first CP to resect an insulinoma of the pancreatic neck (Figure 5); they reported the technique in 1984 in Enciclopedia Medica Italiana (Figure 6)[5].

To spare normal pancreatic parenchyma, Dagradi and Serio removed an insulinoma from the pancreatic neck by CP. This landmark operation in 1982 marked the first use of CP. These Authors described the resective and reconstructive aspects of the technique.

The original technique included the following steps. After incision of the superior and inferior margins of the pancreas the segment harbouring the lesion was mobilized, and the pancreas was isolated at its superior margin from the splenic artery, ligating and severing some collateral vessels. Subsequently the posterior surface of the pancreas was carefully dissected from the splenic vein in such a way as to avoid vessel injury. After placing marginal stitches on the cephalic and distal sides, the pancreas was transected. The resected pancreatic specimen was sent for pathological analysis to check the resection margins and to confirm the diagnosis by frozen sections. Haemostasis of the two raw surfaces was achieved with 4 or 5/0 stitches. Wirsung's duct of the cephalic stump was sutured selectively with a figure-of-eight stitch. A row of 3/0 non-absorbable interrupted overlapping stitches of the mattress type was along the entire length of the stump and tied. An end-to-end pancreatico-jejunostomy was carried out with a single layer of 3/0 non-absorbable interrupted stitches. The operation was concluded with the construction of an end-to-side jejuno-jejunostomy with a double layer of absorbable stitches, approximately 50 cm distal to the pancreatic anastomosis.

Subsequently Serio and Iacono[18-35] have validated
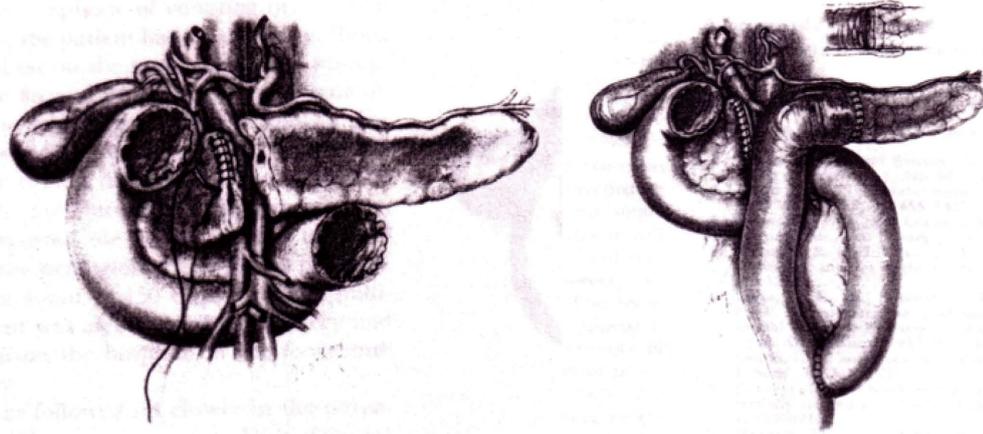
Figure 3 Drawing of reconstruction after pancreatic traumatic transection of the pancreatic neck using by Letton and Wilson (published on *Surg Gynecol Obstet* 109: 473-478, with permission of Elsevier Science Inc. United States).
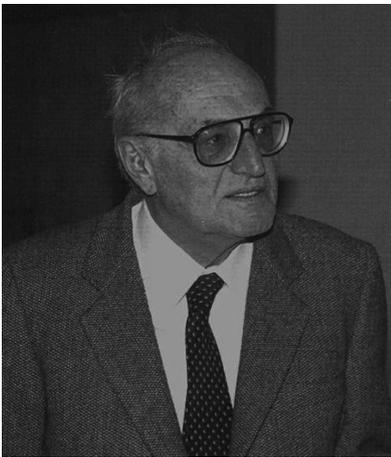

Figure 4 Professor Adamo Dagradi, a pioneer in hepato-biliary-pancreatic surgery (from the book: Scritti in onore di Adamo Dagradi nel XXV anniversario del suo ordinariato, Grafiche Fiorini, Verona 1989 with permission).

Figure 5 Surgical specimen from the first central pancreatectomy operation to resect an insulinoma of the pancreatic neck (published in *J Gastrointest Surg* 11: 364-376 with permission of Springer, New York, United States).

and popularized this technique, with several reports in the national and international literature as well as several congress presentations and videos with the appropriate indications and contraindications.

In these papers the indications for CP were: benign lesions between 2 and 5 cm in size, where a simple enucleation entails risk of injury to the main pancreatic duct; cystic lesions not suitable for enucleation especially in young patients; symptomatic serous cystadenoma; mucinous cystadenoma; pseudopapillary tumours; small tumours that are deeply located in the gland and are therefore not eligible for simple enucleation; focal chronic pancreatitis with isolated and short stenosis of Wirsung's duct; solitary metastases in the pancreatic neck, for example, from kidney, and metastatic pancreatic endocrine tumours in a multimodality program treatment; and selected cases of intraductal papillary mucinous neoplasm (IPMN).

The contraindications of this technique were: malignant tumours (especially ductal adenocarcinoma); neoplastic involvement from other organs (*e.g.,* stomach, or colon); diffuse chronic pancreatitis; large lesions for

which it is not possible to preserve at least 5 cm of distal pancreatic stump; distal body-tail atrophy; and Mellière and Moullé type-III pancreatic vascularisation (the body-tail of the pancreas receives its arterial blood supply exclusively from the transverse pancreatic artery, left branch of the dorsal pancreatic artery)[36].

At the end of the 1980s, this technique aroused criticism owing to the high risk for the formation of fistula because of the presence of two pancreatic stumps.

In 1988, the French surgeon Fagniez reported two cases of limited conservative pancreatectomy[37]. Thereafter Asanuma *et al*[38] reported other two cases of segmental pancreatectomy in 1993, and in the same year Rotman *et al*[39] of Fagniez group, reported 14 cases of medial pancreatectomy.

This operation initially named intermediate pancreatectomy, in 1994 it was renamed as CP[24]. In 1995, the results on 11 patients were presented at Annual Meeting of Pancreas Club Inc. in San Diego by Iacono. Ikeda *et al*[40] published their results with 24 cases, and Fernandez-Cruz and colleagues described three cases (treated with what they called conservative pancreatic resection)[41].

Many different terms to define the same operation
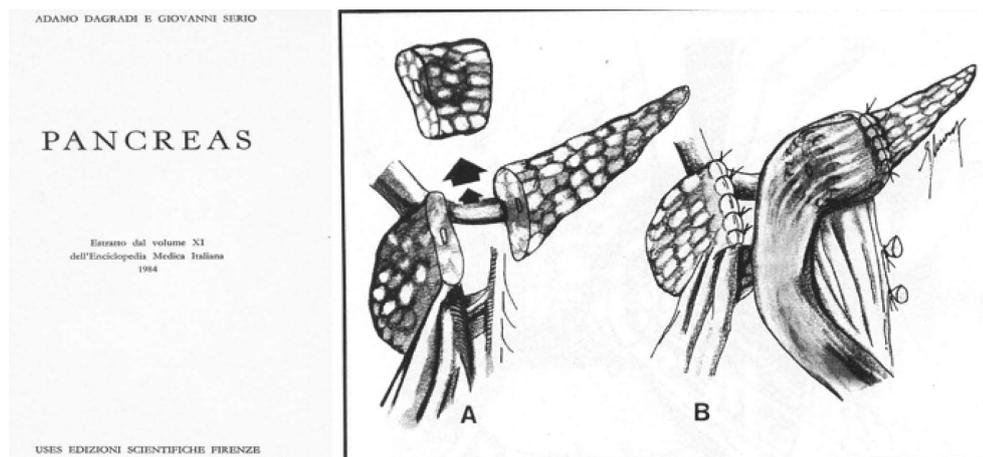
Figure 6 Surgical technique of central pancreatectomy reported in 1984 in Enciclopedia Medica Italiana (with permission of UTET Scienze Mediche, Torino, Italy).
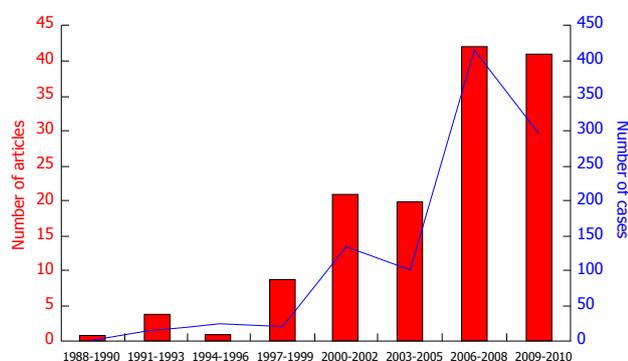

Figure 7 Trend of publications and number of cases of central pancreatectomy from 1988 to 2010.

appears a way to rediscover an already known technique. Among the different denominations the term CP seem to be the most appropriate and nowadays it it the most frequently used in the literature. The other terms that can be currently found are middle pancreatectomy, median pancreatectomy, medial pancreatectomy, segmental pancreatectomy, limited conservative pancreatectomy, intermediate pancreatectomy.

In 1997 we assessed the functional results after CP with oral glucose tolerance test, the pancreo-lauryl test, and assay of faecal fat excretion in all of the operated patients; moreover in a subgroup of patients the same tests were been performed before and after surgery[42]. Our results showed that there was no postoperative impairment of endocrine and exocrine function.

In 1998, Warshaw at Massachusetts General Hospital in Boston applied this technique for the first time in United States and described it in a paper entitled "Middle segment pancreatectomy: a novel technique for conserving pancreatic tissue"[43].

In 1998, Partensky *et al*[44] reported the first 10 cases of reconstruction of the distal stump with pancreatico-gastrostomy. Currently, many pancreatic groups perform CP worldwide[7,45-55].

By the end of the 1990s, the number of publications about CP had increased and to 2010, about 1000 cases have been reported[6,7] (Figure 7).

More recently, in 2003, Baca and Bokan[56] performed the first laparoscopic CP for a case of cystadenoma in a 55-year-old woman. Their full-text original paper was published only in German, while in English was published only the abstract in which they reported the following description: "The patient was placed in lithotomy position. Four trocars were placed the pancreas showed a 6-cm × 6-cm × 6-cm, well-bordered, cystic tumor in the corpus. The healthy tissue in the head area of the pancreas was divided with the linear stapler. The resected pancreas segment was placed in an endobag until removal. The tail segment was anastomized *in situ* end-to-side with the first jejunum loop behind the Treitz's ligament. There was no postoperative complication, and the postoperative course was observed...".

Other authors have described cases managed with a laparoscopic approach[7,57-60]. In 2004, Giulianotti *et al*[61,62] performed the first robot-assisted CP. Since then, other have also undertaken robot-assisted CP[7,62-65]. The description of the operation from the paper of Giulianotti *et al*[62] was: "a 12-mm trocar was placed in order to position a 30-degree-scope robotic camera at the umbilicus. Three 8-mm robotic ports were inserted. An additional 12-mm port was placed on the left side of the umbilicus for use with the assistant's instruments. The first part of the procedure was performed laparoscopically. The gastrocolic ligament was opened. An ultrasonic examination of the pancreas was performed. The da Vinci surgical arm cart was then brought into a position directly cranial to the patient and docked to the robotic trocars. The splenic artery on the superior border of the pancreas was dissected and surrounded with a vessel loop. The superior mesenteric vein was exposed at the inferior edge of the pancreatic neck. Using the robotic grasper, a tunnel was created under the pancreatic neck. Upon completion of the tunnel, tape was passed through, which was

divided to the right side of the lesions by using an endoscopic stapler. Interrupted stitches of polypropylene 4/0 were applied to the proximal stump. The pancreatic body was progressively dissected from the splenic vessels. Each small branch of the splenic vein and artery, to and from the pancreas, was tied and sectioned. Transection of the pancreas was carried out on the left side of the lesions, using a robotic ultracision device. The anterior wall of the distal pancreatic remnant was then anastomosed to the posterior wall of the gastric body, using a single layer of monofilament 4-0 running suture. The gastric wall was and the posterior aspect of the pancreatic stump was sutured to the posterior gastric wall by using a single layer of monofilament 4-0 running suture. Interrupted 4-0 polypropylene stitches were placed to reinforce the pancreaticogastrostomy and to achieve hemostasis of the eventual bleeding point. Two drains were systematically".

In the systematic review, recently published, in 963 CP operations performed before the end of 2010 a complication rate of 45.3% and a mortality rate of 0.8% were reported. The incidence of exocrine and endocrine insufficiency was very low. The most frequent indications for CP were endocrine tumours followed by cystic tumours[66].

From a technical perspective the most frequent reconstruction for the distal stump was pancreaticojejunostomy followed by pancreatico-gastrostomy. On the other hand, the proximal stump is treated in many cases by burying it with interrupted stitches after elective closure of Wirsung's duct[6,7].

Some recent papers have compared CP with DP. A meta-analysis confirmed that the exocrine and endocrine functions are well preserved following CP compared to DP despite a higher incidence of complications associated with CP than DP[6,7].

CONCLUSION

Since 1907 several surgeons applied in the evolution of this technique, the pioneers in the demolitive part (Ehrhardt, Finney, Honjyo) and in the 50s in the reconstructive part (Guillemin and Bessott and Letton and Wilson), only Dagradi and Serio in 1984 published the complete surgical intervention.

Nowadays CP is performed worldwide either by traditional open surgery or by minimally-invasive or robotic approaches. This confirms that the operation does not belong to whom introduced it but to everyone carries out it; however, credit must be given to those from whom it came.

CP is not an alternative to PD or DP. For expert pancreatic surgeons it can be the procedure of choice for benign or low-grade malignant lesions of the neck or proximal body of the pancreas because it does not impair exocrine and endocrine function, even though it increases the risk of postoperative pancreatic fistula.

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Recent studies of 5-fluorouracil resistance in pancreatic cancer

Wei-Bin Wang, Yu Yang, Yu-Pei Zhao, Tai-Ping Zhang, Quan Liao, Hong Shu

Wei-Bin Wang, Yu Yang, Yu-Pei Zhao, Tai-Ping Zhang, Quan Liao, Hong Shu, Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China
Author contributions: Wang WB and Yang Y performed and wrote the paper; Zhao YP designed the study and provided financial support; and Zhang TP, Liao Q and Shu H were involved in reviewing and editing the manuscript.
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Correspondence to: Yu-Pei Zhao, MD, Department of General Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College, No. 1 Shuai Fu Yuan, Dongcheng District, Beijing 100005, China. zhao8028@263.net
Telephone: +86-10-69152600 Fax: +86-10-65295818
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Abstract

Resistance to 5-fluorouracil (5-FU), an important anti-cancer drug, is a serious challenge in the treatment of pancreatic cancer. Equilibrative nucleoside transporter 1 and multidrug-resistance protein (MRP) 5 and MRP8, rather than P-glycoprotein, play important roles in 5-FU transport. Thymidylate synthase, dihydropyrimidine dehydrogenase, methylenetetrahydrofolate reductase and thymidine phosphorylase are four key enzymes involved in 5-FU metabolism. Other metabolic enzymes, including uridine monophosphate synthetase, also contribute to chemoresistance. Intracellular signaling pathways are an integrated network, and nuclear factor kappa-light-chain-enhancer of activated B cells, AKT and extracellular signal-regulated kinases are signaling pathways that are particularly relevant to 5-FU resistance. In addition, recent reports indicate that STAT-3 is a crucial survival protein. Proteomic assays provide a powerful tool for identifying target proteins and understanding the role of microRNAs and stromal factors to facilitate the development of strategies to combat 5-FU resistance.

Core tip: 5-fluorouracil (5-FU) is one of the most important drugs for human pancreatic cancer. Although recent studies have questioned the effectiveness of 5-FU against pancreatic cancer, it remains a good choice for pancreatic cancer. Our paper discusses recent studies that provide novel insights into 5-FU chemotherapy in pancreatic cancer.

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INTRODUCTION

Pancreatic cancer is one of the most fatal types of cancer worldwide, accounting for 3% of new cancer cases and 6% of all cancer-related deaths in the United States[1]. The annual death rate for pancreatic cancer patients has remained stable over the past 10 years, and approximately 4% of patients survive for 5 years after diagnosis[2]. 5-fluorouracil (5-FU), a widely accepted anti-cancer drug, was first introduced in 1957[3]. As a pyrimidine analog, 5-FU exerts its anticancer effects through the inhibition of thymidylate synthase (TS) and the incorporation of its metabolites into RNA and DNA[4,5]. Despite initial doubts

concerning the efficacy of 5-FU, numerous studies have since demonstrated a valuable role for 5-FU in combined treatment protocols compared with single gemcitabine chemotherapy[6,7]. However, 5-FU chemoresistance, which may result from deficient drug uptake, alterations of targets, activation of DNA repair pathways, resistance to apoptosis and the tumor microenvironment, and other serious problems have been reported[8]. In this review, we will discuss recent studies that provide novel insights into the mechanisms of 5-FU resistance.

TRANSPORT MECHANISMS

5-FU targets intracellular enzymes, and thus, the efficiency of 5-FU treatment depends on transport systems. However, there is little information regarding the role of transporters in mediating 5-FU resistance in pancreatic cancer. Nucleoside transporter systems, including human equilibrative nucleoside transporters (hENTs) and concentrative nucleoside transporters (hCNTs), particularly hENT1, play important roles in the cellular uptake and supply of nucleosides and nucleoside analogues. Tsujie *et al*[9] reported that high expression of *hENT1* mRNA led to low sensitivity to 5-FU in pancreatic cancer, which suggests that hENT1 plays an important role in 5-FU resistance and that hENT1 mRNA levels might be a useful marker to predict 5-FU sensitivity in pancreatic cancer. Furthermore, Gao *et al*[10] observed that inhibition of hENT1 by dipyridamole (DP) could increase the intracellular concentration of 5-FU, thereby enhancing cytotoxicity in human pancreatic cancer cell lines. High expression of hENT1 may preferentially facilitate the uptake of nucleosides relative to 5-FU. Alternatively, hENT1 may provide a bilateral channel for 5-FU, whereas other transporters actively pump 5-FU into the cell. For example, 5-FU is a substrate of the human organic anion transporter 2 (hOat2, SLC22A7)[11] but not hCNT1[12]. Members of the ATP-binding cassette (ABC) transporter superfamily facilitate drug resistance *via* their role as efflux pumps. Interestingly, P-glycoprotein (P-gp, ABCB1), which is encoded by the multidrug resistance 1 gene (*MDR1*) and is the most common drug resistance ABC transporter, is not involved in 5-FU resistance[13], but the expression of multidrug-resistance protein 5 (MRP5, ABCC5)[14-16] and MRP8 (ABCC11)[17] is correlated with cellular 5-FU sensitivity.

The role of breast cancer resistance protein (BCRP, ABCG2) remains controversial. ABCG2 can transport the nucleotide CdAMP, similar to several other ATP-binding cassette transporters of the ABCC (multidrug resistance protein) family, and the nucleoside cladribine. In addition, the expression of ABCG2, a target gene of MSX2, correlates with chemoresistance in pancreatic cancer[18,19].

METABOLIC ENZYMES

Previous studies focused primarily on genes involved in 5-FU metabolism. Four intracellular enzymes are considered key determinants in controlling 5-FU sensitivity or resistance: thymidylate synthase (TS, TYMS), dihydropyrimidine dehydrogenase (DPD), methylenetetrahydrofolate reductase (MTHFR) and thymidine phosphorylase (TP)[20]. The majority of studies have focused on gene polymorphisms or expression, and few have examined pancreatic cancer.

5-fluorodeoxyuridine monophosphate (5-FdUMP), the metabolite of 5-FU, directly binds to TS and inhibits its activity, catalyzing the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using 5,10-methylene tetrahydrofolate (CH2THF) as the methyl donor[4]. Immunohistochemical analysis of paraffin-embedded tissues from 212 patients with pancreatic head or periampullary cancer following 5-FU-based adjuvant treatment revealed a significantly increased median survival of patients with low intratumoral TS expression compared with those patients with high TS expression[21]. Furthermore, high TS expression was an independent predictor of poor prognosis. The TS gene can be classified into two different "alleles" based on the expression of a 28-bp variable number tandem repeat (VNTR) in the 5' untranslated region (UTR) of TS: either two repeats (2R) or three repeats (3R) (with three common genotypes, 2R/2R, 2R/3R and 3R/3R)[22]. In addition, the 3R allele can be subclassified according to the presence of a single nucleotide polymorphism (SNP) replacing a cytosine with a guanine (G/C) in 3R (3C or 3G)[23]. An analysis of a panel of seven pancreatic cancer cell lines revealed that cells of the 3R/3R genotype, which express high levels of the TS protein, exhibit lower sensitivity to 5-FU compared with cells of the 2R/2R or 2R/3R genotype[24]. In a clinical study of patients with metastatic gastrointestinal cancer, Cui *et al*[25] observed that patients with the 2R/3R genotype may be more sensitive to chemotherapeutic regimens, including 5-FU, than those with 3R/3R. By contrast, Hur *et al*[26] observed no significant difference in the tumor responses of 3R/3R and 2R/3R patients. In a meta-analysis of 20 studies, Wang *et al*[27] observed a significant increase in the overall survival of rectal cancer patients exhibiting the TS 3R/3R genotype. Taken together, these data highlight the need for further studies investigating the role of TS polymorphisms in 5-FU resistance.

TS, FdUMP and CH2FH4 form an inactive ternary complex that is stabilized by high CH2FH4 levels. MTHFR, a key regulatory enzyme involved in intracellular folate metabolism, converts CH2FH4 to 5-methyltetrahydrofolate (CH3FH4) and reduces 5-FU efficacy. The two most common polymorphisms linked to altered enzymatic activity are C677T and A1298C. In preclinical studies, the C677T mutation was associated with increased chemosensitivity of colon and breast cancers to 5-FU[28], and mutated A1298C variants exhibited enhanced 5-FU efficacy[29]. Clinical studies performed by Delgado-Plasencia *et al*[30] demonstrated that colorectal cancer (CRC) patients expressing variant T genotypes (CT or TT) at the C677T

polymorphism exhibited a higher survival rate after chemotherapy than the homozygote CC variant. No significant associations between the MTHFR c.1298 genotypes or MTHFR diplotypes and survival were observed[31].

More than 80% of administered 5-FU is catabolized by DPD in the liver. Thus, patients with DPD enzyme deficiency are at risk for developing serious 5-FU toxicity. Previous studies have demonstrated an association between DPD expression and patient survival. Immunohistochemical analysis of DPD expression in 176 patients with upper tract urothelial carcinoma (UTUC) revealed no significant association between DPD levels and patient prognosis. However, significantly higher levels of cell growth inhibition and a higher IC50 value for 5-FU were observed in UMUC-3 cells following targeted silencing of DPD by siRNA compared with controls[32]. Ciaparrone *et al*[33] demonstrated that CRC patients receiving adjuvant, systemic 5-FU and exhibiting high DPD expression had significantly shorter disease-free survival and overall survival compared with patients with low DPD expression. An analysis of 15 human pancreatic cancer cell lines and two 5-FU-resistant sub-lines revealed a significant correlation between 5-FU IC50 values and the expression of TS × DPD (quantitative analyses of mRNA expression levels), suggesting that pancreatic cancer cells with high TS and/or DPD levels are more resistant to 5-FU[34].

The first step of activation of 5-FU in tumor tissues involves the conversion of 5-FU to fluorodeoxyuridine by TP. TP, also referred to as platelet-derived endothelial cell growth factor, is an angiogenic factor that promotes angiogenesis *in vivo* and stimulates the *in vitro* growth of a variety of endothelial cells. The role of TP in the clinical response to fluoropyrimidine-based chemotherapy is complex. In a clinical study involving 35 patients with newly diagnosed, locally advanced pancreatic cancer who received radiotherapy with capecitabine, which is metabolized to 5-FU by TP, Saif *et al*[35] revealed that a lower TP/DPD mRNA ratio was significantly associated with higher overall survival. Miyake *et al*[36] also observed this association in a cohort of 25 pancreatic cancer patients following immunohistochemical analysis of the TP/DPD ratio in their surgical specimens.

Furthermore, Griffith *et al*[37] observed differential expression of uridine monophosphate synthetase (UMPS) isoforms in the MIP101 and MIP/5-FU CRC cell lines and demonstrated that a low *UMPS A/B* isoform ratio, rather than the abundance of *UMPS* mRNA, might be predictive of 5-FU resistance.

Taken together, these studies indicate that intracellular nucleoside metabolic enzymes are promising candidates as mediators of 5-FU resistance.

ROLE OF GENES INVOLVED IN CELL CYCLE REGULATION, PROLIFERATION, REPAIR AND APOPTOSIS

DNA and/or RNA damage caused by 5-FU leads to the activation of DNA repair systems or apoptosis. Thus, the alteration of genes involved in cell cycle regulation, proliferation, repair and apoptosis plays an important role in 5-FU resistance.

To investigate genes involved in 5-FU resistance, Wang *et al*[38] performed gene expression analysis using HG-U133A arrays in five breast cancer cell lines, including the 5-FU resistant cell lines MCF-7FU1, MCF-7FU5 and T47DFU2.5 and their drug-sensitive parental counterparts, MCF-7WT and T47DWT. Significant down-regulation of key genes involved in 5-FU activation was observed in 5-FU resistant cells, including *TK*, *UMPK* and *OPRT*. Furthermore, overexpression of genes involved in cell cycle regulation, proliferation, repair and apoptosis, including *TS*, *c-YES*, *NF-ES*, *p65* and *c-Flip*, was detected in the resistant cell lines. Cotransfection of NF-κB p50 and p65 cDNA induced 5-FU resistance in MCF-7 cells and reduced the expression of genes governing the G1-S and S-phase transitions. Cotransfection of NF-κB p50 and p65 cDNA induced 5-FU resistance in MCF-7 cells. Both NF-κB- and 5-FU-induced resistant cell lines exhibited reduced expression of genes governing G1-S and S-phase transitions. The expression of genes involved in DNA replication was also down-regulated in resistant cell lines. These findings were highly consistent with the slower growth rate, higher proportion of G1 cells and lower proportion of S-phase cells in the resistant cell lines. This phenotype may protect resistant cells from cell death induced by the incorporation of 5-FU into DNA chains by allowing time to repair 5-FU-induced damage[38].

Ischenko *et al*[39] tested the hypothesis that inhibition of Src tyrosine kinase could augment the chemosensitivity of the drug-resistant human pancreatic cancer cell lines AsPC5-FU RES and L3.6pl5-FU RES to 5-FU. The authors observed the following: (1) inhibition of Src tyrosine kinase activity by PP2 enhances 5-FU-induced cytotoxicity and induces apoptosis in 5-FU-resistant cells following 5-FU treatment; (2) Src specifically regulates 5-FU chemosensitivity in both the parental and chemotherapy-resistant cell lines; (3) overexpression of TS in chemotherapy-resistant cell lines is suppressed by PP2; and (4) 5-FU-induced EGFR–AKT pathway activation is affected by PP2 in chemotherapy-resistant cell lines, and PP2 restores the chemosensitivity of AsPC5-FU RES pancreatic tumors to 5-FU *in vivo*[39]. Taken together, these studies indicate that 5-FU resistance may be reversed by PP2, a Src tyrosine kinase inhibitor, *via* the EGFR-Akt pathway, by overcoming TS regulation. Zhao *et al*[40] also observed that pERK expression levels were noticeably increased in 5-FU-resistant SW1990/FU cells compared with their parental cell line. Treatment of SW1990/FU cells with the ERK inhibitor PD98059 sensitized cells to 5-FU by activating caspase-8 and reducing phospho-Bcl-2. Yoon *et al*[41] also reported that the AKT and ERK1/2 signaling pathways were activated in the 5-FU-resistant intrahepatic cholangiocarcinoma cell line SCKR. Bcl-2 expression was also elevated in these cells, and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 was capable of altering this phenotype.

Can *et al*[42] demonstrated the importance of a Ca^{2+}-calmodulin (CaM)-p53 axis in 5-FU-induced extrinsic apoptosis. Inhibition of this pathway using a Ca^{2+}-chelator or inhibitors of CaM abrogated the ability of 5-FU to activate caspase-8 and inhibited subsequent cell death. Furthermore, both TS inhibition and misincorporation of 5-FU metabolites into RNA result in p53 stabilization, and p53 may be involved in downstream signaling pathways in response to 5-FU[4].

Dicitore *et al*[43] reported that aberrant constitutive activation of STAT3 protein is frequently detected in pancreatic adenocarcinoma, and type I interferons (IFNs), especially IFN-α, activated the JAK-2/STAT-3 pathway. Dicitore *et al*[44] also reported the therapeutic role of peroxisome proliferator-activated receptor γ (PPAR-γ) in combination with other drugs (IFNs, gemcitabine and COX-2 inhibitors), highlighting molecular interactions and signaling pathways involved in pancreatic cancer cells, including Ras/Raf/MAPK pathway, Akt/PKB signaling, and Erk-1/2 pathway. Vitale *et al*[45] treated human pancreatic cell line BxPC-3 with combination of recombinant IFN-β and PPAR-γ agonist troglitazone, and found a synergistic growth inhibition by MTT assay. Westen blot analysis showed that IFN-β-induced activation of STAT-3, MAPK, and Akt could be counteracted by TGZ-induced inactivation of STAT-3. The combination also decreased anti-autophagic bcl-2/beclin-1 complex formation due to inactivation of the Akt-mTOR-dependent pathway. Spitzner *et al*[46] recently demonstrated that STAT-3 inhibition sensitizes colorectal cancer to 5-FU–based chemoradiotherapy (CT/RT) both *in vitro* and *in vivo*. Inhibition of STAT3 by RNAi-mediated silencing in both SW480 and SW837 cell lines exposed to 3 μmol/L of 5-FU and irradiation, and a subcutaneous xenograft model led to profound CT/RT sensitization. The inhibitory effect of STAT-3 in pancreatic cancer is worth expecting.

Cell signaling networks encompass numerous complicated pathways that involve significant crosstalk. To describe the main molecular mechanisms of 5-FU chemoresistance, we created an illustration based on many related studies; further studies are necessary to elucidate the roles of these networks (Figure 1).

CONTRIBUTION OF STROMAL FACTORS TO DRUG RESISTANCE

Pancreatic cancer cells are typically surrounded by dense stroma. Stromal factors contribute significantly to the tumor microenvironment, but the role of the cancer microenvironment in 5-FU chemoresistance is just beginning to be explored. Sato *et al*[47] tested the sensitivity of MiaPaCa-2 and AsPC-1 cells to 5-FU following pre-incubation with recombinant annexin Ⅱ (rANX Ⅱ). In MiaPaCa-2 cells, treatment with rANX Ⅱ led to the suppression of caspase-3 activation and increased Bcl-2/Bax ratio. Pre-incubation of cells with rANX Ⅱ increased 5-FU resistance. Chen *et al*[48] demonstrated that the ex-

pression of focal adhesion kinase (FAK) related to 5-FU chemosensitivity involves an Akt/NF-kappaB signaling pathway in human CRC cells. Suppression of FAK expression significantly decreased 5-FU resistance and markedly increased the apoptosis of multicellular spheroid culture cells. Thus, 5-FU chemoresistance also requires the FAK/Akt/NF-κB survival signaling pathway. Expression of the obesity hormone leptin[49], stromal cell-derived factor-1α (SDF-1α)/CXCR4 cross-talk[50] and β6-integrin expression[51] have also been associated with 5-FU resistance in colon cancer cells. In addition to these classical signaling pathways, a new membrane receptor, calcium sensing receptor (CaSR), has also been shown to regulate drug resistance. Activation of CaSR by extracellular Ca^{2+} or its agonists enhanced the sensitivity of human colon carcinoma cells to 5-FU and down-regulated TS expression and the anti-apoptotic protein survivin[52]. Furthermore, the tumor-suppressive function of vitamin D in human colon carcinoma cells requires functional CaSR and promotes a cytotoxic response to 5-FU in a CaSR-dependent manner by suppressing the expression of TS and survivin[53]. Recent increased interest in pancreatic stellate cells should provide novel findings related to 5-FU resistance and the tumor microenvironment.

MICRORNAS AND 5-FU RESISTANCE

MicroRNAs (miRNAs) are small, 19-25 nucleotide (nt), non-coding RNAs that function as post-transcriptional regulators capable of blocking the translation of mRNAs into protein and/or promoting the degradation of target mRNAs. Kurokawa *et al*[54] profiled the expression of miRNAs in DLD-1/R and KM12C/R cells, two 5-FU-resistant colon cancer sub-lines derived from the DLD-1 and KM12C cell lines, using Agilent human miRNA microarrays (G4471A) that included 723 human and 76 human viral miRNAs from the Sanger miRBase release 10.1. The authors identified the specific up-regulation of eight miRNAs in DLD-1/R cells and 22 miRNAs in KM12C/R cells, in particular, miR-19b and miR-21. Subsequent miRNA: mRNA immunoprecipitation (RIP)-Chip analysis demonstrated that 66 mRNAs were recruited following the transfection of miR-19b into DLD-1 and DLD-1/R cells, including SFPQ (splicing factor proline and glutamate-rich), which has been linked to cell cycle function. SFPQ functions at different cell cycle stages to maintain sister chromatid interactions[55], and depletion of this gene has been shown to cause abnormal cell accumulation in the S phase of the cell cycle[56]. Similarly, Rossi *et al*[57] observed up-regulation of miR-19a (a paralog of miR-19b) and miR-21 in HT29 and HCT-119 colon cancer cells in response to 5-FU exposure. The majority of miR-21 targets are tumor suppressors, including PTEN[58,59], PDCD4[59] and Bcl-2[59]. By performing *in silico* analysis coupled to experimental validation, Boni *et al*[60] determined that miR-192 and miR-215 target TYMS expression in CRC cell lines; however, down-regulation of TYMS by miR-192/215 did not sensitize CRC cell lines to 5-FU treatment. Based on these results, the au-
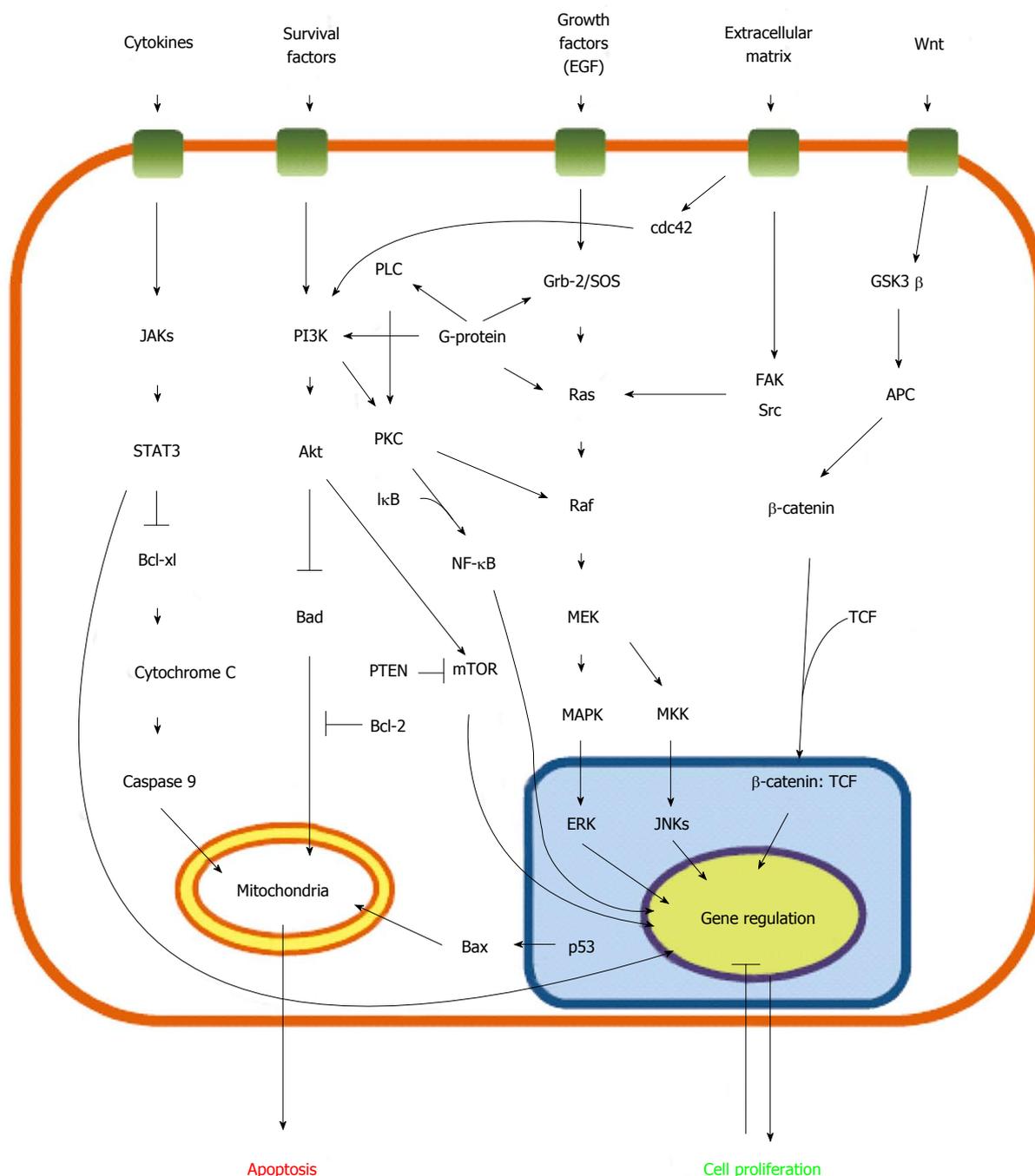
Figure 1 Pancreatic cancer cell survival pathways in 5-fluorouracil resistance. DNA and/or RNA damage caused by 5-fluorouracil (5-FU) leads to the activation of DNA repair systems or the apoptosis cascade. Several cell survival pathways, including the epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, Akt/mechanistic target of rapamycin (mTOR) pathway, STAT3 dependent pathway, phosphatidyl inositol 3-kinase (PI3K)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway and Wnt/GSK3β/β-catenin pathway, are involved in 5-FU resistance in pancreatic cancer.

thors hypothesized that additional events induced by the knockdown of proteins targeted by miR-192 are likely involved in the cellular response to 5-FU independent of TYMS inhibition. Ectopic expression of miR-192 in RKO and LoVo cells transfected with siRNA targeting TYMS decreased 5-FU chemosensitivity. The functions of other miRNAs are currently under investigation, and the use of online databases will likely be critical in understanding their roles in mediating 5-FU resistance[61].

Proteomic investigation of 5-FU resistance

Proteomics is a powerful tool for detecting and identifying drug resistance-related proteins. Two-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS), such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), is now a commonly employed method. A significant number of studies have investigated the mechanisms underly-

ing gemcitabine resistance in pancreatic cancer cells[62-64], and similar studies related to 5-FU resistance are now underway. Yoshida *et al*[65] identified 40 differentially expressed protein spots between TS-1-resistant cells, PK45p and KLM-1, and TS-1-sensitive cells, Panc-1, BxPC-3, MiaPaCa-2 and PK59, using 2-DE and LC-MS/MS. TS-1 is a mixture of 5-FU and tegafur (FT), a metabolically activated prodrug of 5-FU. Among the 40 differentially expressed proteins, 29 were up-regulated, including hypoxia up-regulated protein 1 (oxygen regulated protein, ORP150) and annexin A1. Kimura *et al*[66] identified two ribosomal proteins, L15 and L37, by proteomic analysis of DLD-1 and DLD-1/5-FU cells by 2-DE and MALDI-TOF/TOF-MS/MS. Tan *et al*[67] identified 102 unique proteins, including p16, Maspin, PRDX6, PSMB7, MYL6, PHB and HSP27, in the altered hepatocellular carcinoma (HCC) cell line SMMC-7721/5-FU compared with parent cells. Furthermore, down-regulation of PRDX6 and PSMB7 enhanced 5-FU sensitivity in SMMC-7721/5-FU cells. Isobaric tags for relative and absolute quantitation (iTRAQ) is a non-gel-based technique used to quantify proteins from different sources in a single experiment followed by LC-MS/MS[68]. Using this technique, Tong *et al*[69] identified 52 proteins that were differentially expressed in the HCC cell line BEL7402/5-FU compared with its 5-FU-sensitive counterpart, BEL7402. Of these 52 differentially expressed proteins, 26 were increased in BEL7402/5-FU, notably annexin A3 (ANX3), one of the least-studied members of the annexin family. Importantly, suppression of ANX3 led to the enhancement of 5-FU sensitivity in BEL7402/5-FU cells. Although these experiments are associated with inherent technical variability, proteomic studies provide new targets for investigating novel mechanisms of 5-FU resistance.

CONCLUSION

More than 60 years after its development, 5-FU continues to be an important anticancer drug. However, more significant studies are required to understand the mechanisms underlying 5-FU resistance in pancreatic cancer. Screening at the genomic and proteomic levels has provided an abundance of candidate targets, and summarizing these studies and applying this knowledge for the development of successful 5-FU-based treatment strategies are essential. With comprehensive databases, the analysis of signaling networks, protein-protein interactions and intracellular-extracellular crosstalk is now possible. Multi-drug resistance studies have increased interest in drug-directed research. The study of chemoresistance mechanisms is likely to promote the application of novel, successful combination chemotherapy protocols for improved outcomes for cancer patients.

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High intensity focused ultrasound: A noninvasive therapy for locally advanced pancreatic cancer

Feng Wu

Feng Wu, Institute of Ultrasonic Engineering in Medicine, Chongqing Medical University, Chongqing 400016, China
Author contributions: Wu F solely contributed to this paper.
Correspondence to: Feng Wu, MD, PhD, HIFU Unit, The Churchill Hospital, Oxford University Hospitals, Headington, Oxford OX3 7LJ, United Kingdom. feng.wu@nds.ox.ac.uk
Telephone: +44-1865-763100 Fax: +44-1865-751222
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Abstract

The noninvasive ablation of pancreatic cancer with high intensity focused ultrasound (HIFU) energy is received increasingly widespread interest. With rapidly temperature rise to cytotoxic levels within the focal volume of ultrasound beams, HIFU can selectively ablate a targeted lesion of the pancreas without any damage to surrounding or overlying tissues. Preliminary studies suggest that this approach is technical safe and feasible, and can be used alone or in combination with systemic chemotherapy for the treatment of patients with locally advanced pancreatic cancer. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications. This review provides a brief overview of HIFU, describes current clinical applications, summarizes characteristics of continuous and pulsed HIFU, and discusses future applications and challenges in the treatment of pancreatic cancer.

Core tip: Prognosis in unresectable locally advanced pancreatic cancer is extremely poor. Standard treatments are currently limited to chemotherapy, radiotherapy, or a combination of the two. Though few regimens may offer a limited survival benefit, novel treatment strategies are urgently needed. As a noninvasive approach, high intensity focused ultrasound therapy can selectively ablate a targeted lesion of the pancreas. Preliminary studies indicate that this approach is safe and feasible, and can be used alone or in combination with chemotherapy. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications.

INTRODUCTION

Carcinoma of the exocrine pancreas is the fourth leading cause of cancer-related death in the United States and the Western world. In 2013, 45220 estimated new cases were diagnosed for the United Sates, with 38460 associated deaths[1,2]. Because of the frequent delay in diagnosis, more than 80% of patients have locally advanced or metastatic disease at presentation, and are unsuitable for curative surgical resection[1,2]. Prognosis in pancreatic cancer is generally dismal. Median survival for locally advanced disease is just 6-10 mo, but this falls to 3-6 mo in patients with metastatic disease; overall 5-year survival rate is about 5%[1,2].

Standard options available for treating patients with unresectable pancreatic cancer are limited to chemothera-

py, radiotherapy, or a combination of the two. Gemcitabine is the most commonly used chemotherapeutic agent in pancreatic cancer, and recent studies have shown that a combination of gemcitabine with other chemotherapy agents may offer a limited survival benefit in patients with locally advanced pancreatic cancer[3,4].

As so few patients with pancreatic cancer are suitable for curative surgery and most have only a limited response to chemotherapy, high intensity focused ultrasound (HIFU) has been recently investigated as a potential additional therapy with the intention of tumor debulking and symptom control. Using an extracorporeal approach, it employs focused ultrasound energy to raise the temperature between 56 ℃ and 100 ℃ in a targeted tumor while ultrasound beam is transmitted into a pancreatic lesion, leading to a complete destruction of all the targeted pancreatic cancer cells, instead of local tumor removal[5]. The main advantages of HIFU therapy are less invasive with no incision, no scarring, cheap, less pain and short recovery time. These result in an associated reduction in mortality, morbidity, hospital stay, cost and improved quality of life for cancer patients. The purpose of this article is to review recent developments in the use of HIFU therapy for pancreatic cancer, and to discuss its potential in this application.

DEFINITION OF HIFU ABLATION

Ultrasound is a form of vibrational wave. It can be brought to a tight focus at a distance from its source while an ultrasound beam propagates harmlessly through living tissues. Just as energy in the sun can be concentrated to a point, and used to set fire to combustible material using a magnifying glass, the power of an ultrasound beam can be focused. If the concentrated energy is sufficient, there may be tissue destruction solely within the focal volume, while cells lying elsewhere remain unharmed.

Ultrasound energy absorption by living tissue can result in measurable temperature rises. For HIFU, the energy is greatest within the focal volume, and thus the temperature is maximal there. The mechanism for cell killing is primarily thermal. The temperature rises rapidly, and is held in excess of 56 ℃ for 1 s or longer. This causes immediate coagulation necrosis of the targeted volume. The extent of cellular thermal damage is determined both by the temperature achieved, and the length of time for which it is maintained, the higher the temperature, the shorter the time required to produce identical effects. The boundary of the thermally necrosed region, referred to in HIFU as the "lesion" represents the "56 ℃ for 1 s or longer" contour. Higher temperatures will have been reached at its centre, and in reality, the temperature within the focal volume may rise rapidly above 80 ℃ during HIFU treatments[6,7]. A steep temperature gradient exists at the lesion boundary, and therefore a sharp demarcation between the treated and normal extra-focal tissue is only less than the size of 10 cells, which is histologically observed under light microscope[8,9].
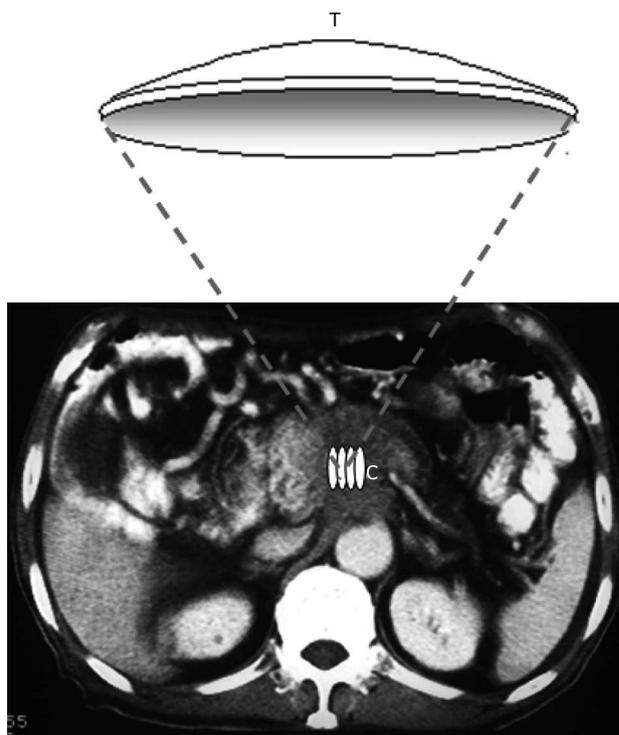

Figure 1 Schematic diagram demonstrating the principle of high intensity focused ultrasound treatment for pancreatic cancer. Ultrasound beam is focused into a small volume in which ultrasound energy is converted into heat to induce the required coagulation necrosis of a targeted pancreatic tumor. T: HIFU transducer; C: The targeted pancreatic cancer.

At high ultrasound intensity levels, not only thermal effects, but those resulting from mechanical mechanisms become important[10]. The most important non-thermal mechanism for tissue disruption in HIFU fields is acoustic cavitation, which leads to the local destruction of the tissue due to cavitation-induced high pressures and temperatures[9].

The intention of a HIFU treatment is to deliver ultrasound energy to a well-defined targeted volume at depth, and to induce complete coagulation necrosis of the tumor. A single (1-3 s) HIFU exposure usually produces a very small cigar-shaped lesion of dimensions of 10-20 mm along the beam axis and 1-2 mm in the transverse direction. However, by placing lesions side by side, conformal confluent volumes of ablation of clinically relevant size can be achieved, as shown in Figure 1. It is important that individual lesions overlap in order that no viable tumor cells remains between them. Due to the nature of using a small lesion to cover the large volume of tumor, theoretically there should be no limitation of tumor size, but it will take long and costly treatment times when attempting to ablate a large tumor. For safety reasons, in weaken and old patients HIFU procedure may be divided into two sessions when tumor is too large, and each session ablates the separated part of the targeted tumor. However, as HIFU is guided by either US or MRI, it is unsuitable to treat small tumors (less than 0.5 mm), if they are not clearly detected by both images.
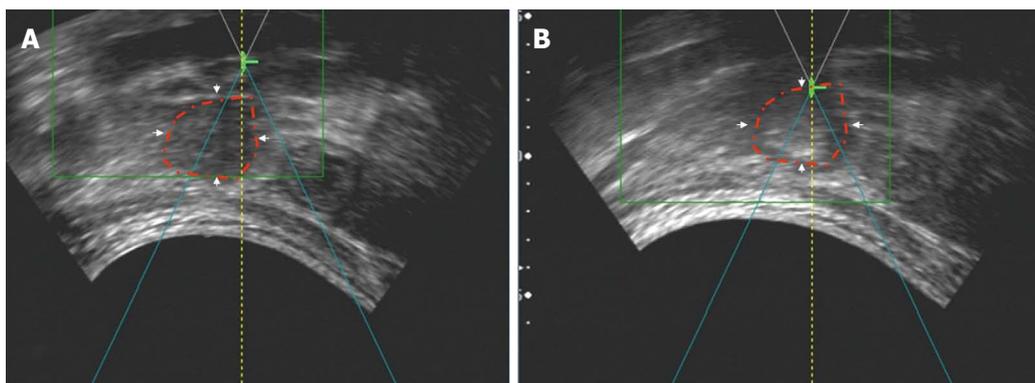
Figure 2 Grey-scale changes in a treated pancreatic cancer on real-time ultrasound images during high intensity focused ultrasound exposure. A: Ultrasound (US) image obtained before high intensity focused ultrasound (HIFU) shows a pancreatic cancer lesion present in the body of the pancreas (arrowheads); B: US image obtained immediately after the one-slice HIFU treatment shows the obvious hyperechogenicity of the treated pancreatic tumor (arrowheads).

DESTRUCTIVE EFFECTS OF HIFU ABLATION

Direct thermal and non-thermal effects

The effects of thermal ablation on a targeted tumor are determined by increased temperatures due to thermal energy deposition, rate of removal of heat, and the specific thermal sensitivity of the tissue. As the tissue temperature rises, the time required to achieve irreversible cellular damage decreases exponentially. At temperatures between 50 ℃ and 55 ℃, cellular death occurs instantaneously in cell culture[11]. Protein denaturation, membrane rupture, cell shrinkage, pyknosis, and hyperchromasia occur *ex vivo* between 60 ℃ and 100 ℃, leading to almost immediate coagulation necrosis[12]. In addition, acoustic cavitation, one of the mechanical effects induced by HIFU ablation, is the most important non-thermal mechanism for tissue disruption[10]. Small gaseous nuclei existing in subcellular organelles and fluid in tissue are the sources of cavitation, which can expand and contract under the influence of the acoustic pressure. During the collapse of bubbles, the acoustic pressure, shear stress, and subsequently high temperature can induce the local destruction of a targeted tissue[13].

Thermal effects on tumor blood vessels

Structural and functional changes are directly observed in tumor blood vessels after thermal ablation[14-17]. These changes are not as well described as thermal effects on the tissues, but they rely on varying temperatures. At temperatures between 40 ℃ and 42 ℃, there is no significant change in tumor blood flow after 30-60 min exposure[18]. Beyond 42 ℃ to 44 ℃, there is an irreversible decrease in tumor blood flow, with vascular stasis and thrombosis, resulting in heat trapping and progressive tissue damage[19]. When temperatures exceed 60 ℃, immediate destruction of tumor microvasculature occurs[20]. It cuts the blood supply to the tumor directly through the cauterization of the tumor feeder vessels, leading to deprivation of nutrition and oxygen. Thus, tissue destruction can be enhanced by the damage caused by thermal ablation to tumor blood vessels.

CLINICAL OUTCOMES

Up to now, HIFU has been largely reported as a palliation option to treat patients with locally advanced pancreatic cancer. There are mainly two HIFU commercial devices available to clinical application, and the HIFU-treated patients are almost from Asia. Both devices incorporate B-mode ultrasonography to target and monitor the therapeutic procedure. One is Chongqing HIFU system (Model-JC and JC200 HIFU system, Haifu Medical Technology, Chongqing, China). It is an extracorporeal ultrasound-guided HIFU device, and employs continuous HIFU wave with high intensity (5-20 kW/cm^2). The therapeutic regime is a typically thermal ablation, and each patient receives HIFU treatment only once. Treatment time is dependent on the size of a targeted tumor, which ranges from 45 min to 3.2 h. During the procedure, acoustic intensity should gradually increase in the focus until a hyperechogenic change is clearly observed within the targeted lesion on ultrasound imaging (Figure 2). This tissue response is not only a good real-time imaging assessment to determine whether coagulation necrosis could occur during each HIFU shot in the targeted tumor, but also a imaging feedback to control energy delivery of HIFU exposures. Chongqing HIFU device got CE approval in 2005 for the treatment of pancreatic cancer, and now it has been increasingly used for clinical applications in Europe. The other is a FEB-BY Serial HIFU System (China Medical Technologies, Beijing, China). It is also an extracorporeal ultrasound-guided HIFU device, but uses pulsed-wave HIFU with low intensity (< 3 kW/cm^2). The therapeutic regime is similar to focused ultrasound hyperthermia treatment. Each patient has separately undergone 4-7 sessions over the course of 10-14 d, and every session lasts about 1-1.5 h. During the procedure, acoustic intensity should drop down if a patient feels abdominal pain or discomfort. The clinical outcomes of the both HIFU devices are summarized in Tables 1 and 2.

Table 1 Studies of continuous-wave high intensity focused ultrasound treatment for patients with advanced pancreatic cancer

Study	n	Patients	Treatment method	HIFU Device	Outcome and survival	Complications
Wu et al[21]	8	A phase I-II study of HIFU for advanced pancreatic cancer, unresectable. Average tumor size 5.89 cm (4.5-8 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 8/8 (100%); Median survival: 11.25 mo (2-17 mo)	None
Orsi et al[22]	6	Late-stage pancreatic cancer, unresectable. Average tumor size 4.6 ± 1.4 cm	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 6/6 (100%); Median survival: 7 mo; Overall survival: 42.9% at 12 mo and 21.4% at 24 mo	Portal vein thrombosis: 1/6 (16%)
Wang et al[24]	40	Advanced pancreatic cancer, unresectable. Average tumor size 4.3 cm (2-10 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 35/40 (87.5%) Median survival: 8 mo (stage III: 10 mo; stage IV: 6 mo); Overall survival: 58.8% at 6 mo and 30.1% at 12 mo	None
Sung et al[25]	46	Advanced pancreatic cancer, unresectable. Average tumor size 4.2 ± 1.4 cm (1.6-9.3 cm)	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	A significant reduction of pain score ($P < 0.001$); Median survival: 12.4 mo; Overall survival: 52.2% at 6 mo, 30.4% at 12 mo, and 21.79% at 18 mo	Mild abdominal pain: 16/46 (34%); severe abdominal pain with vomiting: 2/46 (4%); transient fever: 3/46 (6%); 2nd-3rd skin burns: 2/46 (4%); pancreaticoduodenal fistula: 1/46 (2%),; gastric bleeding due to ulcer: 1/46 (2%)
Wang et al[26]	224	Advanced Pancreatic cancer	One-session HIFU monotherapy	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief and survival data not reported	Abdominal distension, anorexia and nausea: 10/224 (4%); asymptomatic vertebral injury: 2/224 (1%); obstructive jaundice: 1/224 (1%)
Gao et al[27]	39	Locally advanced pancreatic cancer, unresectable. Tumor size unclear	One-session HIFU alone: 14 pts; HIFU + gemcitabine: 25 pts	Continuous HIFU irradiation, Model-JC HIFU System	Pain relief: 31/39 (79.5%) Median survival: 11 mo; Overall survival: 82.1% at 6 mo, and 39.5% at 12 mo	None
Zhao et al[28]	37	A phase II study of HIFU + gemcitabine for locally advanced pancreatic cancer, average tumor size 3.4 cm (1.7-8.5 cm).	Gemcitabine on days 1, 8 and 15, and multiple HIFU sessions on days 1, 3 and 5. The combined treatment repeated every 28 d	Continuous HIFU irradiation, HIFUNIT-9000 HIFU System	Overall survival: 12.6 mo (95%CI: 10.2-15.0); Pain relief: 29/37 (78%)	Fever: 26/37(70%); neutropenia: 6/37 (16%); thrombocytopenia 2/37 (5%); nausea and vomiting 3/37 (8%); diarrhea 2/37 (5%)

HIFU: High intensity focused ultrasound; pts: Patients.

Continuous-wave HIFU treatment

The first success of HIFU ablation for advanced pancreatic cancer was conducted in Chongqing China in 2000[21]. It was a phase I-II prospective clinical trial, and both survival benefit and pain control were observed during follow-up period. Eight patients with locally advanced pancreatic cancer were treated only once with continuous-wave HIFU alone for palliation. The tumor ranged from 4.5 to 8 cm in diameter (mean 5.89 cm), and was mainly located in the body and tail of the pancreas. The results showed that HIFU treatment was safe and feasible, and no complications were recorded. After HIFU, pre-existing severe back pain of presumed malignant origin disappeared in each patient. Follow-up images showed reduction or absence of tumor blood supply in the treated region with significant shrinkage of the ablated tumor, as shown in Figure 3. Of them, 4 patients died (median survival time 11.25 mo, range 2-17 mo), and the remaining 4 patients were still alive with median

follow-up time of 11.5 mo (range 9-16 mo). The authors concluded that HIFU could be safe, effective and feasible in the treatment of patients with advanced pancreatic cancer.

Subsequently, several clinical studies were performed to investigate the safety and feasibility of HIFU for the treatment of patients with advanced-stage pancreatic cancer[22-25]. They were one-arm phase I-II trails, and clinical results were very encouraging, as shown in Table 1. Orsi et al[22] reported a preliminary experience of using HIFU for 6 patients with unresectable pancreatic cancer. After treatment, either PET/CT or contrast-enhanced MR images showed complete ablation in 5 of 6 patients, and pain relief was observed in all patients. Median survival was 7 mo, and 1- and 2-year survival rates were 42.9% and 21.4% respectively. Local skin burn was not observed, but portal vein thrombosis was detected as a major complication in one patient after treatment. The same group also treated 2 inoperable patients with pancreatic

Table 2 Studies of pulsed-wave high intensity focused ultrasound treatment for patients with advanced pancreatic cancer

Study	n	Patients	Treatment Method	HIFU Device	Outcome and Survival	Complications
Wang et al[29]	15	Late-stage pancreatic cancer, unresectable, average tumor size 5.6 cm (2.2-8 cm)	Multiple-session HIFU monotherapy, average sessions 8.1 (2-12)	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief: 13/13 (100%) No survival data available	Mild abdominal pain: 2/15 (13%)
Li et al[30]	25	Advanced pancreatic cancer, unresectable, average tumor size unclear	One-session HIFU: 19 pts; 2-session HIFU: 6 pts; average sessions 1.2	Pulsed HIFU irradiation, FEB-BY HIFU System	Performance statue and pain improvement: 23/25 (92%); median overall survival: 10 mo; 1-year survival: 42%	First-degree skin burn: 3/25 (12%)
Ge et al[31]	20	A retrospective study for unresectable pancreatic cancer, average tumor size (4.5 ± 1.2) × (3.5 ± 1.0) cm	Multiple-session HIFU monotherapy; average HIFU session 9.3 ± 4.1	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief and survival data not reported	Mild abdominal pain: 5/25 (25%); subcutaneous fat callus: 4/25 (20%); 2nd-degree skin burn: 1/25 (5%); pancreatic effusion: 1/25 (5%)
Xiong et al[32]	89	A retrospective study for unresectable pancreatic cancer. Tumor size not reported	Multiple-session HIFU monotherapy: 84 pts; HIFU + gemcitabine: 5 pts; HIFU sessions ranging 4-10	Pulsed HIFU irradiation, FEB-BY HIFU System	Pain relief: 54/67(80%), median survival: 26.0 mo (stage Ⅱ), 11.2 mo (stage Ⅲ) and 5.4 mo (stage Ⅳ)	Superficial skin burns: 3/89 (3%); subcutaneous fat sclerosis: 8/89 (6%); asymptomatic pseudocyst: 1/89 (1%)
Lee et al[33]	12	Advanced pancreatic cancer, unresectable, average tumor size 3.5 cm (2.3-5.3 cm)	Multiple-session HIFU monotherapy: 9 pts; HIFU + gemcitabine: 3 pts; average HIFU sessions: 4.2 (1-18)	Pulsed HIFU irradiation, FEB-BY HIFU System	Median survival for those receiving HIFU alone: 10.3 mo; Overall survival for 3 patients receiving the combined treatment: 26.0, 21.6 and 10.8 mo, respectively	Pancreatitis: 1/12 (8%); skin burn: 5/12 (41%); subcutaneous fat sclerosis: 2/12 (16%)

HIFU: High intensity focused ultrasound; pts: Patients.

neuroendocrine tumor (insulinomas)[23]. Both patients suffered from episodes of severe nightly hypoglycemia, which was not efficiently controlled by medication. During 9-mo follow-up, local disease control and symptom relief were achieved in them without any complications. Wang et al[24] followed up HIFU-treated 40 patients with advanced pancreatic cancer (stage Ⅲ, 13 patients; stage Ⅳ, 27 patients). Average tumor size was 4.3 cm (range 2-10 cm). After HIFU, pain relief was achieved in 87.5% of the patients. The median overall survival was 8 mo for all patients, including 10 mo in stage Ⅱ and 6 mo in stage Ⅲ patients. Six-month and 1-year survival rates were 58.8% and 30.1% respectively. No severe complications were observed during follow-up period.

Sung et al[25] treated 46 patients with advanced pancreatic cancer, including 18 in stage Ⅲ and 28 in stage Ⅳ disease. Average tumor size was 4.2 ± 1.4 cm (range: 1.6-9.3 cm). After HIFU treatment, contrast-enhanced MR images showed 90%-100% ablation in 38 lesions, 50%-90% in 8 and within 50% in 3 lesions. Pain score (visual analog scale) was significantly reduced from 4.9 ± 1.1 to 2.1 ± 1.1 ($P < 0.001$). Overall median survival from initial diagnosis was 12.4 mo. Overall survival rates at 6, 12, and 18 mo from HIFU were 52.2%, 30.4%, and 21.79%, respectively, with a median survival of 7.0 mo. Minor complications (abdominal pain, fever and nausea) was observed in 28 (57.1%) of 49 HIFU treatment. Major complications were detected in 5 (10.2%) of 49 treatment, including 2-3 degree skin burn in 2, pancreaticoduodenal fistula in 2 and gastrointestinal tract

bleeding due to gastric ulcer in one patient. The authors concluded that HIFU was safe and effective, and it could induce excellent local tumor control in most patients with advanced pancreatic cancer.

The largest clinical experience of using HIFU treatment for advanced pancreatic cancer was reported by Wang et al[26]. A total of 224 patients were enrolled in this study for safety analysis of HIFU treatment. Gastrointestinal dysfunction such as abdominal distension and anorexia with slight nausea was observed in 10 cases (4.5%) after HIFU treatment. One case with pancreatic head cancer developed obstructive jaundice 2 wk after HIFU treatment. Vertebral injury, identified by MRI, occurred in 2 cases, although no symptoms were seen. No severe complications were observed in all enrolled patients. These results indicated that HIFU was a safe, noninvasive treatment. However, no long-term follow-up and survival data were reported in this study.

HIFU combined with chemotherapy was also used to treat advanced pancreatic cancer. Gao et al[27] reported an initial use of HIFU alone or HIFU plus gemcitabine for the treatment of 39 patients with locally advanced pancreatic cancer. Among them, 14 patients received one-session HIFU monotherapy, and the remaining 25 patients underwent HIFU combined gemcitabine therapy. After treatment, no server complications were observed, and pain relief was achieved in 31 (79.5%) of 39 patients who had previous pain. Medial overall survival was 11.0 mo, and 6- and 12-mo survival rates for all patients were 82.1% and 39.5% respectively. However, medial survival
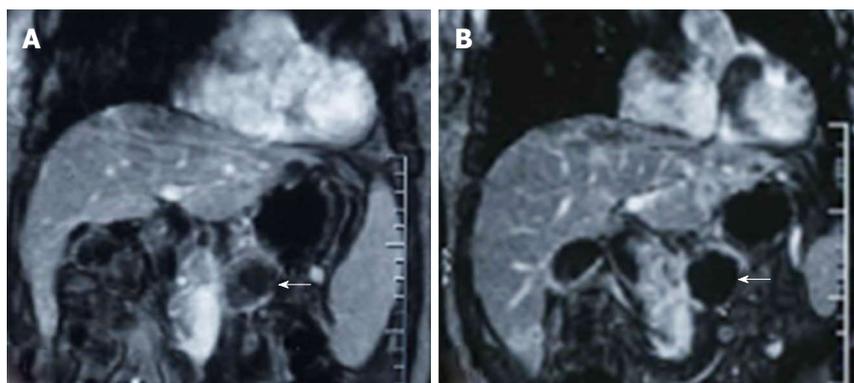
Figure 3 Contrast-enhanced T-weighted MR images obtained in a patient treated with high intensity focused ultrasound for advanced pancreatic cancer. The tumor was 4.5 cm in diameter and located in the body of the pancreas. A: Image obtained before high intensity focused ultrasound (HIFU) shows blood supply in the pancreatic lesion (arrows); B: Image obtained 2 wk after HIFU shows no evidence of contrast enhancement in the treated lesion (arrows), which is indicative of complete coagulation necrosis in the treated pancreatic cancer.

and 1-year survival were significantly higher in patients treated with HIFU plus chemotherapy while compared with those in patients treated with HIFU alone. There were statistical differences between two groups ($P < 0.01$). Zhao et al[28] also reported a phase Ⅱ trial investigating the safety and efficacy of concurrent gemcitabine and HIFU for the treatment of 37 patients with locally advanced pancreatic cancer. The average tumor size was 3.4 cm (range 1.7-8.5 cm). All patients received gemcitabine 1000 mg/m^2 on days 1, 8, and 15, and concurrent HIFU treatment (HIFUNIT-9000, AS Sci-Tech, Shanghai, China) on days 1, 3, and 5. The combined treatment regime was repeated every 28 d and continued until disease progression, patient refusal, or an unacceptable toxicity. The results showed that overall survival was 12.6 mo (95%CI: 10.2-15.0), and the estimates of overall survival at 12 and 24 mo were 50.6% (95%CI: 36.7%-64.5%) and 17.1% (95%CI: 5.9%-28.3%), respectively. Pain was relieved in 22 (78.6%) of 28 patients who had complained of abdominal pain consistent with tumor-related pain. After treatment, grade 1 or 2 fever was detected in 70.3% of patients. Six patients (16.2%) experienced grade 3/4 neutropenia, and 2 (5.4%) had grade 3 thrombocytopaenia was documented. Grade 3 nausea/vomiting and diarrhea were observed in 3 (8.1%), and 2 (5.4%) patients respectively. The authors concluded that concurrent gemcitabine and HIFU was a tolerated treatment modality with promising activity in patients with previously untreated locally advanced pancreatic cancer.

Pulsed-wave HIFU treatment

Compared to continuous-wave HIFU treatment, pulsed HIFU usually uses low energy with a multiple-session treatment regime. The first study of pulsed HIFU for advanced pancreatic cancer was reported by Wang et al[29] in 2002, and 15 patients received multiple-session pulsed HIFU treatment for the purpose of palliation. HIFU session ranged from 2 to 12 (average 8.1). The average tumor size was 5.6 cm (range 2.2-8 cm). Seven patients had a lesion located in the head of the pancreas, including 4 who had previously received gallbladder-intestine bypass

operation. The remaining 8 patients had carcinoma of the body and tail of the pancreas. After HIFU, pain relief was observed in 13 (100%) of 13 patients who had previously cancer-related pain. Tumor size shrank in 3 patients while the other 12 patients had no change. Unfortunately, there were no survival benefit data available in this study. Mild abdominal pain was recorded as a complication in 2 of 15 patients.

Li et al[30] reported a clinical result of pulsed HIFU for the treatment of 25 patients with unresectable pancreatic cancer. Of them, 19 patient received one-session HIFU, and the remaining 6 had two session treatments. The treatment time was less than 60 min in each session. After HIFU treatment, 3 patients had first degree skin burn, but they recovered without any medication. Performance statue and pain improvement were observed in 23 (92%) of 25 patients during follow-up period. Overall average survival time was 10 mo, and 1-year survival rate was 42% for all patients. Ge et al[31] analyzed clinical results of HIFU treatment for advanced pancreatic cancer in a retrospective study. Twenty patients received multiple-session HIFU treatment, and the average number of HIFU sessions was 9.3 ± 4.1 for each patient. After treatment, mild abdominal pain was observed in 5 (25%) patients, and subcutaneous fat callus was found in 4 (20%) of 25 patients. One patient experienced 2nd-degree skin burn, and pancreatic effusion was also detected in 1 patient. However, no pain relief and survival data were reported in this study.

Xiong et al[32] reported the largest retrospective study of using pulsed HIFU treatment for advanced pancreatic cancer. Eighty-nine patients with pancreatic cancer were analyzed after HIFU, including 4 in stage Ⅱ, 39 in stage Ⅲ, and 46 in stage Ⅳ disease. Tumors were located in the pancreatic head in 34 patients (38.2%), and in the body and/or tail of the pancreas in 55 patients (61.8%), although tumor size was unclear. In order to treat an entire volume of the tumor, 4-10 HIFU sessions were needed for each patient. After treatment, pain relief was achieved in 54 (80.6%) of 67 patients who had pain prior to HIFU. The median survival was 26.0 mo in stage Ⅱ patients,

11.2 mo in stage III, and 5.4 mo in stage IV patients. Complications included superficial skin burns (3.4%), subcutaneous fat sclerosis (6.7%), and an asymptomatic pancreatic pseudocyst (1.1%). The authors concluded that although this retrospective study had significant limitations, preliminary results suggested that the clinical application of HIFU for pancreatic cancer appeared to be safe and was a promising modality of treatment for palliation of pain related to pancreatic cancer.

Similar to continuous HIFU treatment, pulsed HIFU combined with chemotherapy were also used to treat advanced pancreatic cancer. Lee et al[33] reported initial experience of using pulsed HIFU for the treatment of 12 patients with unresectable pancreatic cancer, including 9 treated with HIFU alone, and 3 treated with pulsed HIFU combined with gemcitabine. Median tumor size was 3.5 cm (range: 2.3-5.3 cm), and HIFU sessions ranged from 1 to 18 (average 4.8 sessions). After HIFU treatment, skin burn was observed in 5 patients including 1 in 2nd-degree and 4 in 1st-degree skin burn. Subcutaneous fat sclerosis caused by thermal injury was detected in 2 patients, and one patient developed acute pancreatitis with a large pseudocyst after treatment. The median survival for those receiving HIFU treatment alone was 10.3 mo. However, the overall survival of three patients treated by HIFU combined with gemcitabine was 26.0, 21.6 and 10.8 mo, respectively, suggesting that concurrent pulsed HIFU and chemotherapy could be potentially more effective in the treatment of unresectable pancreatic cancer.

DISCUSSION AND CONCLUSION

HIFU is an attractive emerging therapy for unresectable pancreatic cancer. It has been offered as a palliation option for improving the quality of life in patients with advanced-stage pancreatic cancer. Almost all studies have been conducted for the assessment of technical safety and feasibility, and clinical outcome have showed that HIFU therapy is safe and reproducible.

Many of early concerns that surrounded the safety of HIFU treatment for pancreatic cancer have been addressed in the pilot studies. As shown in the Tables 1 and 2, the incidence of complications directly caused by HIFU is relatively lower while compared with radiation therapy and minimally-invasive thermal ablation approaches. Mild complications include abdominal pain, nausea and vomiting, skin burn, and subcutaneous fat sclerosis. They usually occur in 3%-20% patients, and recover in a short time after HIFU treatment, without any medication. Severe complications are observed in 3 patients, including 1 case with portal vein thrombosis, 1 with pancreaticoduodenal fistula, and 1 with obstructive jaundice. Two patients experience pancreatitis with a large pseudocyst around the inflammation site, and 1 patient has gastrointestinal bleeding due to gastric ulcer after treatment. These demonstrate that HIFU is a promising approach with a few adverse effects for the treatment of unresectable pancreatic cancer. However, contraindica-

tions should be considered if a targeted lesion is too close to the duodenum and bile duct. It can extremely increase the risk of bowel perforation and bile leakage because of HIFU damage on these normal structures. Unfortunately, there is no exact safe distance between the tumor and adjacent vital structures available to HIFU treatment currently, and further studies are needed in animal models to define it.

Most clinical results to date are obtained in retrospective studies, and there are a few phase II prospective clinical trials performed in research settings for assessment of HIFU efficacy. These studies have shown that HIFU can significantly improve the quality of life in patients with advanced-stage pancreatic cancer. Pain relief is obviously observed in 78%-100% patients after treatment. Median survival time ranges from 7 to 12 mo, which is dependent on the TNM stage of disease. Case reports reveal that while HIFU is combined with chemotherapy (gemcitabine), median survival and overall survival rate seem better than HIFU alone, but this claim needs to be confirmed in randomized, two-arm clinical trials. In addition, almost all studies uses symptom relief, survival and MRI/CT changes as evidences of assessing treatment effects on pancreatic cancer, instead of histomorphological examination following HIFU treatment. Further studies are needed to investigate the characteristics of histological changes in pancreatic cancer after HIFU treatment.

Two various regimes of therapeutic strategy have been noticed in HIFU treatment. One is continuous HIFU, and the other is pulsed HIFU treatment. They are totally different in both technical parameter and therapeutic strategy, as shown in Table 3. Using high intensities ranging from 5 to 20 kW/cm^2, each continuous HIFU shot can induce coagulation necrosis of a targeted tumor. It is a one-session treatment, and can be used alone for the treatment of unresectable pancreatic cancer. There is no need to be repeated if the tumor is significantly ablated. In addition, the appearance of a hyperechoic region of in the focus is clearly observed on ultrasound imaging immediately after each shot, as shown in Figure 2. Either sedation or general anesthesia is required for patients during treatment procedure due to discomfort and pain. After treatment, the patients require hospitalization for several days.

In contrast, pulsed HIFU uses lower ultrasound intensities, which is usually less than 3 kW/cm^2. It is a multiple-session treatment, and needs to be repeated for many times ranging from 5 to 10 sessions if the patients are suitable. Some patients require sedation during treatment procedure, but most of them don't need it if there is no pain or discomfort. It is a one-day procedure, and there is no need for patients to stay in hospital after treatment. Recent studies have indicated that pulsed HIFU can significantly enhance chemotherapeutic agents against tumor cells[33-36], suggesting that pulsed HIFU may be a treatment approach using focused ultrasound for hyperthermia, instead of HIFU for inducing coagulation necrosis. Actually, focused ultrasound hyperthermia has

Table 3 Technical and therapeutic differences in continuous high intensity focused ultrasound and pulsed focused ultrasound therapy

	US wave	US intensity	Treatment session	Change in US image during procedure	Treatment mechanism	Treatment use	Required anesthesia	Appearances in follow-up images
Continuous HIFU therapy	Continuous	5-20kW/cm²	One session	Real-time hyperechoic change in the focus during each shot	Thermal ablation for coagulation necrosis	Monotherapy, used alone	Sedation or general anesthesia	No contrast enhancement in tumor on MRI/CT; negative uptake on PET/CT
Pulsed focused ultrasound therapy	Pulsed	< 3 kW/cm²	Multiple sessions	No real-time US image change during each shot	Hyperthermia for enhancing sensitive to chemotherapeutic agents	Need to be combined with chemotherapy	Sedation or none	Tumor shrinkage on MRI/CT; negative uptake on PET/CT

HIFU: High intensity focused ultrasound; US: Ultrasound.

been used as adjuvant to radiotherapy and chemotherapy for cancer treatment in the 1990s[37,38]. It can raise the temperature of the tumor from 37 ℃ to 42-45 ℃ for 60 min. This may make some cancer cells more sensitive to radiation and chemotherapy, or harm other cancer cells that both therapies cannot damage[39,40]. It is obvious that focused ultrasound hyperthermia uses lower acoustic energy to heat tumor, and there is no coagulation necrosis that occurs in the treated tumor while compared with HIFU treatment. However, HIFU is a therapeutic approach to locally heat and destroy diseased tissues through thermal ablation. In order avoid any confusion related to the definition of HIFU and hyperthermia, it is highly recommended to use pulsed focused ultrasound hyperthermia rather than pulsed HIFU treatment in the future.

In conclusion, HIFU ablation has been shown a promising approach for the palliative treatment of advanced pancreatic cancer. The nature of non-invasiveness and highly treatment precision has made HIFU become more attractive emerging therapy. It has much potential for further clinical investigation and technical improvements. Currently, preliminary studies suggest that this approach is technical safe and feasible, and can be used alone or in combination with systemic chemotherapy. It can effectively alleviate cancer-related abdominal pain, and may confer an additional survival benefit with few significant complications. However, large, prospective, multi-center randomized clinical trials will be needed to assess the long-term efficacy, and determine the future role of this technique for the treatment of locally advanced pancreatic cancer. Once oncologic efficacy data from those trials are available, HIFU ablation will become an attractive treatment option for patients with pancreatic cancer.

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2014 ADVANCES IN PANCREATIC CANCER

Endoscopic ultrasonography for surveillance of individuals at high risk for pancreatic cancer

Gabriele Lami, Maria Rosa Biagini, Andrea Galli

Gabriele Lami, Maria Rosa Biagini, Andrea Galli, Gastroenterology Unit, Department of Clinical Pathophysiology, University of Florence Medical School, 50139 Florence, Italy
Author contributions: All authors contributed equally to the preparation, writing and editing of this article; all authors read and approved the final manuscript.
Correspondence to: Andrea Galli, Professor, Gastroenterology Unit, Department of Clinical Pathophysiology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy. a.galli@dfc.unifi.it.
Telephone: +39-05-54271419 Fax: +39-05-54222409

Abstract

Pancreatic cancer is a highly lethal disease with a genetic susceptibility and familial aggregation found in 3%-16% of patients. Early diagnosis remains the only hope for curative treatment and improvement of prognosis. This can be reached by the implementation of an intensive screening program, actually recommended for individuals at high-risk for pancreatic cancer development. The aim of this strategy is to identify pre-malignant precursors or asymptomatic pancreatic cancer lesions, curable by surgery. Endoscopic ultrasound (EUS) with or without fine needle aspiration (FNA) seems to be the most promising technique for early detection of pancreatic cancer. It has been described as a highly sensitive and accurate tool, especially for small and cystic lesions. Pancreatic intraepithelial neoplasia, a precursor lesion which is highly represented in high-risk individuals, seems to have characteristics chronic pancreatitis-like changes well detected by EUS. Many screening protocols have demonstrated high diagnostic yields for pancreatic pre-malignant lesions, allowing prophylactic pancreatectomies. However, it shows a high interobserver variety even among experienced endosonographers and a low sensitivity in case of chronic pancreatitis. Some new techniques such as contrast-enhanced harmonic EUS, computer-aided diagnostic techniques, confocal laser endomicroscopy miniprobe and the detection of DNA abnormalities or protein markers by FNA, promise improvement of the diagnostic yield of EUS. As the resolution of imaging improves and as our knowledge of precursor lesions grows, we believe that EUS could become the most suitable method to detect curable pancreatic neoplasms in correctly identified asymptomatic at-risk patients.

Core tip: In the era of early diagnosis and screening programs, endoscopic ultrasound (EUS) represents the most promising tool able to identify pancreatic precursor neoplasms in high risk individuals. If compared to other imaging techniques, it is highly accurate to diagnose small pancreatic cancer and pre-malignant lesions, with very low rate of complications and limitations. Here are reported the current role of EUS in various international screening programs and its future possible developments.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the western world[1,2], with a median age at diagnosis of 71 years and 45220 new cases and 38460 deaths in 2013 in the United

States[3]. In contrast to other causes of cancer death (lung, colorectal, breast and prostate), which have declined in the last years, the death rate from PDAC has increased during the same time period[4]. It is a highly aggressive tumor characterized by an incidence rate almost equaling the mortality rate and an overall 5-year survival of approximately 5%-6%[1,2]. This dismal prognosis is mainly due to the fact that the tumor is characterized by a locally advanced or metastic stage at the presentation, low resection rates and poor response to radiotherapy and chemotherapy.

Even though complete resection improves median survival, at the time of diagnosis only 10% to 25% of pancreatic cancer patients will be amenable to potentially curative resection[5]. Also in this case 5-year survival remains low (10% to 24%)[6,7].

However, longer survival has been reported for complete resection of early stage tumors thus identifying patients who have early, small, localized tumors at presentation could improve this poor overall survival rate[8].

Resection of small tumors (< 2 cm or T1) improves 5-years survival (30% to 60%)[9,10]. However it has been alluded that the better prognosis is for tumors < 1 cm (T1a) with 5-years survival up to 78%[6,11,12].

To date, however, it might be difficult to detect such a small pancreas cancer, mainly due the fact that more than 90% of PDAC measuring 1 cm or less in diameter are asymptomatic.

Probably the only way to improve survival lies in identifying early disease or precursor lesions through a screening program of asymptomatic individuals.

As premalignant stages of disease have been identified, and the sensitivity of pancreatic imaging has improved with endoscopic ultrasound (EUS) and high-resolution magnetic resonance imaging (MRI), early detection of small curable pancreatic cancers and premalignant lesions now seems possible[13-16].

Unfortunately, due to the overall low incidence of the disease, accounting for 3% of all new cancer cases in the United States and a life-time risk of 1.3% in the general population, and the lack of simple, safe, accurate, inexpensive, and non-invasive diagnostic tests for early lesions, a widespread screening program does not seem feasible at present.

Multiple risk factors for pancreatic cancer development have been identified like male gender, obesity, African-American or Ashkenazi Jewish descent, nickel exposure, smoking, lack of physical activity, and calorie intake[17-20].

Beside them, also members of a family with a strong history of disease or individuals with inherited pancreatic cancer syndromes, carrying a known genetic mutation, should be considered at high risk of developing pancreatic cancer (high risk individuals, HRIs)[21-25]. Screening of these high-risk groups seems to be of benefit since genetic susceptibility and familial aggregation are responsible of 3%-16% of pancreatic cancers[26-28].

These individuals can be divided into two groups: those who belong to families in which pancreatic cancer affects at least two first-degree relatives without a known genetic mutation (familial pancreatic cancer, FPC) and those with hereditary syndromes or diseases that predispose to the development of pancreatic cancer (Table 1).

FAMILIAL PANCREATIC CANCER

The former represents the largest proportion of hereditary PDAC.

Prospective studies demonstrated an increased risk of pancreatic cancer in healthy first degree relatives (FDRs), related to the number of family members affected. This risk has been estimated to be 2.3 to 4.5-fold greater in individuals with one FDR with pancreatic cancer, 6.4-fold greater in individuals with two FDRs with the disease and 32 to 57-fold greater in individuals with three or more FDRs affected[29-32].

Similarly to other familial tumors, the median age of presentation in patients with FPC is up to 20 years earlier than in patients with sporadic cancer (49 years *vs* 61 years)[33-35] with an "anticipation phenomenon" in the affected kindred and a trend to become more severe and appear at an earlier age as the disorder is passed from one generation to the next[35,36]. Currently, the genetic etiology of most cases of FPC remains undetermined but complex segregation analysis of these patients has led to the discovery of various candidate pancreatic cancer susceptibility genes such as BRCA2 (6%-17% of cases)[37,38], partner and localizer of BRCA2 (PALB2) (1%-4% of cases)[39,40] and palladin, even if mutations of the latter have been identified in normal controls as well[41-43].

Due to the complex nature of pedigrees, a Mendelian risk prediction tool for PDAC, named PancPRO was developed in 2007.

This is a prediction model for FPC that, using full pedigree data and age of family members, estimates the probability that an asymptomatic individual will develop the disease[44].

INHERITED PANCREATIC CANCER SYNDROMES

Individuals with certain tumor syndromes have a marked increase in risk of developing pancreatic ductal adenocarcinoma.

These syndromes are represented by familial atypical mole-multiple melanoma, Peutz-Jeghers syndrome, hereditary pancreatitis, cystic fibrosis, familial breast-ovarian cancer, hereditary non-polyposis colorectal cancer, familial adenomatous polyposis, Li-Fraumeni syndrome.

Familial atypical mole-multiple melanoma

Familial atypical mole-multiple melanoma (FAMMM) is an autosomal dominant disease associated with mutations within CDKN2A gene (p16 Leiden)[45,46]. Its inactivation is associated with PDAC that was found 13 to 38-fold more frequent than expected[46,47], with a cumulative risk

Table 1 Genetic diseases associated with pancreatic cancer risk			
Risk condition	Relative risk	Risk by age 70	Gene
Familial pancreatic cancer			*PALLD*
1 first-degree relative	2.3-4.5	2%	*BRCA2*
2 first-degree relatives	6.4-18	3%	*PALB2*
⩾ 3 first-degree relatives	32-57	16%	
Familial atypical multiple mole melanoma	13-38	15%-20%	*CDKN2A/p16*
Peutz-Jeghers Syndrome	132	11%-60%	*STK11/LKB1*
Hereditary pancreatitis	50-87	30%-75%	*PRSS1*
			PRSS2
			SPINK1
			CTRC
Cystic fibrosis	5.3	<5%	*CFTR*
Familial breast ovarian cancer	3.5-10	5%	*BRCA2*
	2.3-3.6	1%	*BRCA1*
Hereditary non-polyposis colon cancer	2.3-8.6	3%-4%	*MLH1*
			MSH2
			MSH6
Familial adenomatous polyposis	4.5-5	2%	*FAP*
			MUTYH
Li Fraumeni sindrome	Unknown	Unknown	*TP53*

by age 75 of 15% to 20%[48,49].

Peutz-Jeghers syndrome

Peutz-Jeghers syndrome (PJS) is an autosomal dominant genetic disease characterized by an increased risk of various neoplasms, including pancreatic cancer[50,51] and it is often associated with mutations within *STK11* gene, a tumor suppressor gene. Patients with PJS have a 132-fold increased risk[50] and an 11%-36% cumulative risk of developing PDAC with an early age of onset (average: 40.8 years)[50,52]. In this kind of patients, it frequently develops through IPMN[23,53].

Hereditary pancreatitis

Hereditary pancreatitis (HP) is an inherited form of chronic pancreatitis characterized by mutations within *PRSS1*, *PRSS2*, *SPINK1*, *CFTR* and *CTRC* genes[54,55]. PDAC is often a consequence of this condition[56,57] insomuch so resected pancreata from patients with HP frequently demonstrated PanIN-3 lesions (50%)[58]. Patients with hereditary pancreatitis have a 53 to 87-fold increase risk[57,59] with an age of onset at 50 years in smokers[60]. Lifetime risk is 30% to 75% in patients with paternal inheritance[57,59].

Cystic fibrosis

Cystic fibrosis (CF) is a disorder associated with mutations within CFTR gene with an increased risk for PDAC (5.3-fold)[61], in fact the histological aspect of CF associated lesions is very similar to that of "classical" chronic pancreatitis, characterized by atrophy of acinar tissue, fibrosis, and inflammation[62,63].

Familial breast-ovarian cancer

Familial breast-ovarian cancer (FBOC) is an autosomal

dominant inherited disease due to mutations within *BRCA1* or *BRCA2* genes.

The risk of PDAC among BRCA1 mutation carriers is low (2.3-3.6 fold than general population)[64,65]. Conversely BRCA2 mutation carriers had a 3.5-10-fold increased risk[66,67] and a 5% lifetime risk of pancreatic cancer[67].

Hereditary non-polyposis colorectal cancer

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant genetic condition due to the inherited mutations in DNA-mismatch repair genes, such as *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM*[68]. The estimated relative risk of pancreatic cancer is 2.3 to 8.6-fold higher with a lifetime risk of pancreatic cancer (3%-4%)[69,70]. Carriers of MLH1 mutations have a higher risk than carriers of MSH2 (5.6 *vs* 2.3)[71].

Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is an autosomal dominant disease of the colon caused by mutations within the gene *APC*. Among FAP pediatric carriers, pancreatoblastoma may represent an extracolonic manifestation of FAP[72]. The relative risk for pancreatic cancer is 4.5 in patients with the syndrome[73] and the lifetime risk 2%[74].

Li-Fraumeni syndrome

PDAC seems to be a part of the cancer spectrum of the Li-Fraumeni syndrome (LFS), a disease caused by mutations within *TP53* gene[63,75]. It is has been estimated that about 1.3% of these patients show pancreatic cancer[63,76].

PRECURSOR LESIONS

The ideal screening method for HRIs should detect small asymptomatic pancreatic cancers and, mainly, benign non-invasive precursor lesions, to allows for curative surgical resection[77,78]. In fact pancreatic carcinogenesis should be intended as a multistep phenomenon with progressive changes from the normal pancreatic ductal epithelium to infiltrating carcinoma[79].

The other three well known precursor lesions are: pancreatic intraepithelial neoplasms (PanINs), intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs)[78-81].

Pancreatic intraepithelial neoplasia

PanINs are usually asymptomatic and are characterized by microscopic papillary or flat, noninvasive epithelial neoplasms that are usually < 5 mm in diameter and confined to the pancreatic ducts[78,82].

According to the degree of cytological and architectural atypia, PanINs are divided into three grades[83]: PanIN-1: minimal atypia; flat (PanIN-1A) and papillary types (PanIN-1B); PanIN-2: moderate atypia; PanIN-3: severe atypia.

The evidence that this kind of lesions are linked to invasive carcinoma is based on clinical associations and

genetic analysis[81,84-86].

Mucinous cystic neoplasms

Mucinous cystic neoplasms (MCNs) are cystic epithelial neoplasms that occur almost in women, lack of communication with the pancreatic ductal system and have a predilection for the body and tail[80,87].

Malignancy rates of resected MCNs vary from 6% to 36%[80] and usually resembles common ductal adenocarcinoma.

Intraductal papillary mucinous neoplasms

Intraductal papillary mucinous neoplasms (IPMNs) are a more aggressive neoplasm compared to MCNs. They represent a disorder of the pancreatic ductal system, characterized by cystic dilatation. Clinically, three different varieties exist: main duct type characterized by diffuse dilatation of the main pancreatic duct, branch duct type (IPMN-BD) appearing as dilatation of branch ducts, and mixed-type involving both of them.

These lesions are thought to undergo transformation from adenoma to borderline neoplasms, and finally to carcinoma, similarly as seen with PanINs.

Patients with IPMN-MD have a risk of malignancy of approximately 50%-90%[16,86-89], *vs* 6%-46% in patients with IPMN-BD[16,87,89,90]. In these patients, the risk of malignancy increases with presence of symptoms, mural nodules and size over 3 cm[89]. IPMNs are mainly present in familial pancreatic cancer kindred and in PJS and FAP patients where seems to have a more aggressive biological behavior (increased growth rate and degeneration) compared to sporadic IPMNs[22,91]. IPMNs are more prevalent in high risk individuals than in the general population (16%-42% *vs* 0.2%)[92], moreover they are commonest in specimens from FPC than in sporadic PDAC (33% *vs* 6%)[81].

SCREENING

The goal of screening could be the reduction of pancreatic cancer-related mortality. As previously reported, surrogate end point in pancreatic cancer could be the identification and resection of potentially curable lesions (high-grade precursors and early invasive carcinomas). There is no evidence that diagnosing these lesions will improve survival, but there are data suggesting that resection of very early disease is associated with better prognosis[93,94]. However, no consensus opinion could be reached on the best suitable approach for screening until available imaging modalities and biomarkers will become adequate to detect early stage cancer. Actually, serum markers, computed tomography, magnetic resonance (MRI) ± cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography and endoscopic ultrasound haven't all the features of an effective screening tool[95-100]. Describing the screening modalities is beyond the aim of this review. Whatever the approach a surveillance program should be recommended for patients with a risk of

PDAC development greater than 10-fold[22,23,77].

This degree of risk includes family members with ≥ 3 first-degree relatives with pancreatic cancer and patients with hereditary pancreatitis, FAMMM and PJS.

A screening test should also be performed in individuals with syndromes associated with pancreatic cancer and known high-risk factors, such as cystic neoplasia, duct ectasia, diabetes mellitus, smoking history and chronic pancreatitis[101]. To evaluate the risk to develop pancreatic cancer can be used mathematical models, such as the PancPRO model (see above).

No clear consensus was achieved on when to start screening. It seems reasonable to start at 40-50 years of age (30 years for PJS) or 10-15 years earlier than the younger kindred affected by pancreatic cancer[21,22,96,102].

There is no consensus also on the frequency, because evidence on the natural history and rate of progression of pancreatic cancer in high risk patients is still lacking. However, yearly screening seems to be the most suitable approach[21,22,36,103] even if some centers recommend 3 years intervals in case of negative screening exam and absence of other risk factors associated. A more aggressive protocol can be used for patients with abnormal findings at the last screening[52]. In these cases a subsequent screening could be done every 3-6 mo[22,103] or every 3-12 mo[21,36,100].

The majority of studies have generally used the same imaging test for surveillance as for baseline screening, while others suggest an alternating use of MRI/MRCP and EUS[36,98](Figure 1).

ROLE OF ENDOSCOPIC ULTRASONOGRAPHY

Endoscopic ultrasonography (EUS) is known as a powerful imaging tool for studying pancreatic diseases. In particular it has been described as a very accurate imaging technique for early detection of pancreatic cancer providing high-resolution images of the pancreas without the risk of radiation exposure and identifying mural nodules (focal thickening of the wall in branch duct IPMNs), which are associated with increased risk of malignancy[16,82]. With its high resolution, in experienced hands it is able to detect focal lesions as small as 2-5 mm[22,104-106] with the possibility of taking bioptic samples by fine needle aspiration (FNA) for histopathological examination. EUS has been described as a highly sensitive method for pancreatic malignancy[107], but results for accuracy differ. Early studies have shown a better accuracy in detecting PDAC for EUS compared with dual phase helical CT (97% *vs* 73%, respectively)[108]. This results were also confirmed when EUS was compared with multiphase helical CT (98% *vs* 86%, respectively)[107,109]. The prospective CAPS3 study is the first blinded study that compared standardized pancreatic protocol CT, secretin-enhanced MRI/MRCP and EUS for one-time screening in HRIs. It showed that EUS and MRI are better than CT for the detection of small, cystic, pancreatic tumors, with a diag-
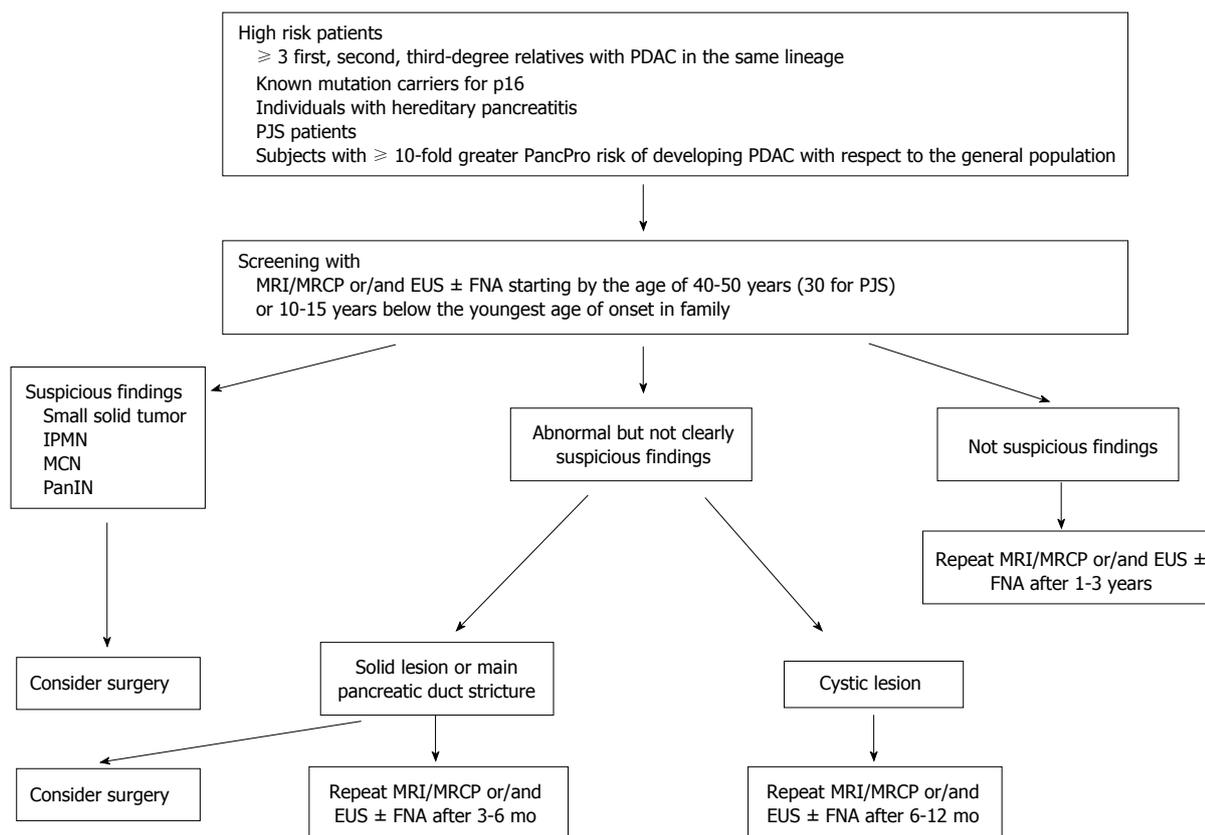
Figure 1 Management algorithm for individuals at risk of pancreatic cancer. EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; CT: Computed tomography; FNA: Fine needle aspiration; PDAC: Pancreatic ductal adenocarcinoma; PJS: Peutz-Jeghers syndrome; MRI: magnetic resonance imaging; MRCP: Magnetic resonance cholangiopancreatography; IPMN: Intraductal pancreatic mucinous neoplasia; MCN: Mucinous cystic neoplasm; PanIN: Pancreatic intraepithelial neoplasia.

nostic yield of 42.6%, 33.3% and 11%, respectively[110]. EUS was also found to be superior to MRI and CT in sensitivity regarding the detection of IPMN-derived and -concomitant PDACs at the first examination (100% *vs* 53% and 53% and 61% *vs* 33% and 39%, respectively) and during a 5 years follow-up period (100% *vs* 50% and 56%, respectively)[111]. In this setting EUS detected PD-CAs significantly better than the other modalities and it appears to be more useful than CT and MRI for the early detection of pancreatic cancer (Table 2).

Another recent study[112] has shown an incremental increase in diagnostic yield of EUS-FNA over CT (36%) and MRI (54%) for prediction of a neoplastic cyst and an increase in overall accuracy for diagnosis of neoplastic pancreatic cysts by the addition of EUS±FNA.

A normal EUS examination seems to have a high negative predictive value (NPV)[113]. Two recent studies including patients with suspicion of pancreatic cancer followed for 23.9 and 25 mo, respectively, showed that none of those with a normal EUS evaluation developed pancreatic cancer (NPV = 100%)[114,115].

Furthermore, EUS-guided fine needle aspiration (EUS-FNA) may provide a histological diagnosis of cancer and a means of detecting dysplasia in precancerous lesions[23]. A recent meta-analysis has demonstrated that EUS-FNA is highly sensitive (89%), specific (96%), accurate (97%) and has a very good positive likelihood

ratio (16.08) and an acceptable negative likelihood ratio (0.13)[116]. Moreover, another recent study not included in the meta-analysis previously reported[117], confirmed these values and has shown that the diagnostic accuracy of EUS-FNA could be further improved by the addition of pancreatic juice analysis.

EUS complications are rare and the risk of perforation is similar to standard upper endoscopy (< 0.03%). Also EUS-FNA of pancreatic lesions can be considered a safe technique, especially if several technical points are taken into account in each specific situation the endosonographer perform a FNA[118]. The two major complications after a FNA are pancreatitis (0%-2%)[119,120] and bleeding (0% to 1.3%)[121,122], while the risk of infection exists only when mucinous cystic lesions are involved[118]. No deaths were reported[120-123].

Actually, the diagnosis of PanINs by imaging tests is very challenging. The surgical resection of early curable neoplasms detected during screening programs in at-risk individuals has permitted to study the morphology of unadulterated precursor lesions in this kind of patients[21,81]. In particular: (1) PanINs are frequently associated with lobulocentric atrophy and fibrosis; and (2) PanINs are often multifocal.

The combination of these alterations produces grossly appreciable changes in the pancreas with a mosaic of fibrosis, atrophy and uninvolved parenchyma, very similar

Table 2 Endoscopic ultrasound-based studies on screening for individuals at risk for pancreatic cancer

Ref.	No. of patients	High-risk groups	Imaging test	Target lesions	Diagnostic yield	Limits of the study
Brentnall *et al*[21]	14	FPC	EUS + ERCP + CT	PanIN ⩾ 2	50%	
Kimmey *et al*[104]	46	FPC	EUS	PanIN ⩾ 2	26%	
Canto *et al*[22]	38	FPC, PJS	EUS	IPMN, PC	5.30%	Low PPV
Canto *et al*[23]	78	FPC, PJS	EUS	IPMN, PC, PanIN ⩾ 2	10.20%	
Poley *et al*[135]	44	FPC, PJS, FAMMM	EUS	IPMN, PC	22.70%	No pathological confirmation of IPMN
Langer *et al*[103]	76	FPC, FAMMM	EUS + MRCP	IPMN	1.30%	Moderate risk patients
Verna *et al*[162]	51	FPC, FBOC	EUS and/or MRCP	IPMN, PC, PanIN ⩾ 2	12%	
Schneider *et al*[36]	72	FPC, FAMMM	EUS + MRCP	IPMN	12.50%	No pathological confirmation
Canto *et al*[110]	216	FPC, FBOC, PJS	EUS + CT + MRCP	IPMN, PC	39%	Mainly no pathological confirmation

FPC: Familial pancreatic cancer; PJS: Peutz-Jeghers syndrome; FAMMM: Familial atypical multiple mole melanoma; FBOC: Familial breast ovarian cancer; EUS: Endoscopic ultrasonography; ERCP: Endoscopic retrograde cholangiopancreatography; CT: Computed tomography; MRCP: Magnetic resonance chol angiopancreatography; PanIN: Pancreatic intraepithelial neoplasia; IPMN: Intraductal pancreatic mucinous neoplasia; PC: Pancreatic cancer; PPV: Positive predictive value.

to chronic pancreatitis[81,124].

These quite subtle ductal and parenchymal changes are often detectable by EUS using standard criteria for the diagnosis of chronic pancreatitis, such as heterogeneity, multifocal lobularity, echogenic foci, hypoechoic nodules, strands and dilated main and branch pancreatic ducts[22,124,125].

In literature, chronic pancreatitis-like changes are found in variable rates. The John Hopkins group detected these findings in 45% and 61% of the examined HRIs in whom they were significantly more common, compared with control subjects, regardless of age and alcohol exposure[22,23]. This ultrasonographic diagnosis of chronic pancreatitis was surgically confirmed in all but one of the HRIs who underwent surgery. Furthermore, all but 1 of these patients had branch duct-type IPMNs[22]. In the University of Washington study, the authors suggested that the pancreatitis-like changes, which are part of the phenotype of FPC kindreds, are expression of un underlying pancreatic dysplasia rather than chronic pancreatitis[21]. Finally the German group reported a relative low prevalence (22.4%) with all but one normal findings at MRI/MRCP evaluation[103].

These studies suggest that features of chronic pancreatitis should be noted during screening because although the precursor lesions may be too small to visualize by currently available imaging technologies, the effects they produce such as cysts and nodules in a background of intact parenchyma, can be detected by EUS in the hands of an experienced operator.

This was also confirmed in IPMNs. In a recent study conducted on forty patients, who underwent resection for IPMN, PanIN was researched on surgical specimens and the pathological data were compared with endosonography features. EUS changes corresponded to PanIN lesions in 83% of cases and it was able to detect 69% of patients with PanIN lesions (57% of those with panIN-3)[126].

Nevertheless, the presence of a chronic pancreatitis drastically reduces the diagnostic value of EUS, because of the intraductal and parenchymal changes associated with chronic inflammation and fibrosis could not to be differentiated from premalignant pancreatic lesions[127].

In summary the clinical significance of these changes in HRIs remains unclear. They may be indicative of a precursor lesion of PDAC, but these data must be carefully assessed.

Another field of application for EUS in HRIs is in differentiation between focal pancreatitis and pancreatic cancer. Contrast enhanced EUS seems to be a promising technique due to perfusion characteristics of microvessels[128]. Hocke *et al*[129] analyzed the sensitivity and specificity for the diagnosis of pancreatic carcinoma of conventional endoscopic B-mode, power Doppler ultrasound and contrast-enhanced power mode. They reported an increase from 73.2% to 91.1% and from 83.3% to 93.3% respectively, with the use of contrast-enhanced power mode *vs* conventional EUS. The major limits of EUS are: (1) high interobserver variety, even among experienced endosonographers, especially for diagnosis of pancreatitis like changes[130,131]; (2) the need for sedation because of the minimally invasive nature of the procedure; (3) the need of additional clinical and imaging information[112] to improve accuracy as demonstrated by Meining *et al*[132] who reported a worse overall accuracy for a strictly blinded EUS examinations (61.1%) compared to the accuracy of routine and unblinded evaluation with additional imaging information (72.2% and 75.0%, respectively); (4) Low sensitivity in case of chronic pancreatitis, diffusely infiltrating cancer and a recent episode of acute pancreatitis[133,134]; and (5) Low availability outside major centres.

Currently, many international screening protocols are available throughout the world and the majority of them use EUS as the main imaging tool for screening, because of its ability to detect masses < 1 cm[21-23,132,135], with CT or MRI/MRCP scans and ERCP proposed in combination with EUS[136].

The first EUS-based screening program was prospectively conducted by Brentnall *et al*[21] at the Washington University, on a small group of 14 high-risk patients from three unrelated pancreatic cancer kindred that had two

or more affected members in at least two generations. The study evaluates an EUS- and ERCP-based approach with the aim to detect pancreatic cancer precursor lesions (PanINs). The EUS and ERCP suspected signs of PanINs were no specific chronic pancreatitis-like changes. Seven patients (50%) had an abnormal EUS and ERCP histological confirmed as precancerous changes in the pancreas (PanIN-2 and 3) without any invasive cancer.

A follow up study of the same group confirmed a high yield (26%). It was based on a large cohort of 46 patients and was conducted using EUS as the first diagnostic approach, with ERCP for patients with EUS abnormalities. Twelve patients with imaging abnormalities were referred to histological examination and all of them revealed widespread precancerous lesions (PanIN 2 e 3), without evidence of invasive pancreatic cancer[136].

Canto *et al*[23] screened HRIs for early pancreatic neoplasia with an EUS-based and an EUS- and CT-based[22] prospective controlled study at Johns Hopkins University. In the former approach they used EUS to screen 38 asymptomatic individuals from high risk families (≥ 3 affected relatives and PJS). Six pancreatic lesions were detected: four benign masses and two neoplastic (one adenocarcinoma and one IPMN; screening yield of 5.3%). Either the CT or ERCP evaluations did not detect the single PDAC. In the latter one, pancreatic abnormalities were compared in 78 high-risk individuals (72 from FPC kindred and 6 PJS) and 149 control patients. If the EUS was abnormal, EUS-FNA and ERCP were performed. This approach found 8 patients with pancreatic neoplasms (10.2%) confirmed by surgery or FNA (6 patients had benign IPMNs, 1 had an IPMN with invasive ductal adenocarcinoma and 1 patient had PanIN-3) and no pancreatic neoplasia among the control subjects. All of the lesions visualized by CT were also detected by EUS, while CT missed two IPMNs > 1 cm in the second study and one pancreatic cancer in the first one. Moreover, ERCP correctly diagnosed only 2 of the 7 confirmed IPMNs seen by EUS.

In contrast to these findings, Langer *et al*[103] published their results of a prospective screening study conducted by the National German Familial Pancreatic Cancer Registry (FaPaCa) on 76 individuals from 34 FPC and FAMM kindreds. The protocol included CA 19-9 and CEA serum values, EUS, and MRI combined with MRCP at the screening visit. EUS-FNA was performed in the case of indefinite abnormalities and in case of diffuse parenchymal irregularities. Only three serous cystoadenoma, one IPMN, three PanIN 1 and one PanIN 2 were pathologically confirmed. Three of them, the smaller ones, were detected by EUS, but not by MRI. No cancers were identified and only IPMN was considered a significant precancerous lesion for a diagnostic yield of 1.3%.

This lower yield could be explained by the fact that this study included also a large number of patients at a moderate risk (< 10-fold) with a fraction of high-risk patients of 42% *vs* 55% for the second study of the Johns Hopkins University. Moreover, PanIN 1 e 2 and serous cystoadenoma were not considered precancerous lesions.

During long term follow-up[36] (24 mo-extended surveillance), this study showed histologically proven precancerous or cancerous lesions in 4 individuals (5.5%) and additional branch duct IPMN in 5 ones, with a diagnostic yield of up to 12.5%, close to the previous rates reported by the Johns Hopkins and the Rotterdam groups.

In comparison, Poley *et al*[135], of the Dutch group, published the results of a prospective study using EUS in 44 asymptomatic high risk family members with FPC, BRCA1, BRCA2, or p16 germline mutation carriers, and patients with PJS. They found asymptomatic PDAC in three patients (6.8%, two with lymph node metastases), and seven IPMNs (16%). Their high yield (22.7%) may be related to the selection of known carriers of mutations at high risk to develop pancreatic cancer with a higher fraction of individuals at elevated risk.

Nevertheless, it has to be pointed out that IPMNs in both German study and in the Dutch study are EUS-diagnosis, not histologically confirmed. The 12.5% and 16% results may as well represent overestimations.

COST EFFECTIVENESS

A screening test can be considered successful if the benefits/costs ratio is favourable. As previously reported, a EUS-based screening allows an early diagnosis of PDAC, while it is not still clear if this approach could be considered cost-effectiveness.

Rulyak *et al*[137] compared one-time EUS-based screening to no screening in a hypothetical cohort of 100 members 50 years old of FPC kindred. The life time medical costs and life expectancy were compared, assuming a 20% prevalence of pancreatic dysplasia and 90% sensitivity of EUS and ERCP. They demonstrated that endoscopic screening of these individuals increases patient life expectancy (38 years, similar to other common preventive medical interventions) in a cost-effective manner ($16885 per life-year saved on the base-case ICER, an indicator which take into account the third-part payer and the societal perspectives). Only patients with a pre-test probability of pancreatic dysplasia of 16% or greater and individuals under 70 years of age seem to have benefits from this approach. Moreover, the sensitivity of EUS and ERCP must be at least 85% in order for screening to be effective. The cost-effectiveness of repeated screening was not determined.

In contrast, Rubenstein *et al*[138] have performed a clinical and economic evaluation of EUS for 45 years-old male first degree relatives with chronic pancreatitis diagnosed by EUS on screening exam. They compared 4 strategies: do nothing, prophylactic total pancreatectomy, EUS and EUS-FNA and assessed mortality, quality of life, complications and costs. They addressed the inferiority of EUS compared to a no-screening approach because of the low sensitivity of EUS in the presence of chronic pancreatitis-like changes. EUS-FNA provided intermediate results. The prophylactic total pancreatectomy could be considered the better approach in terms od life expectancy if the lifetime risk of pancreatic cancer is

46% or greater.

These studies are based on one-time screening and so are not applicable to a individuals who require repeated screening examinations during their life. A review conducted by Latchford *et al*[139] focused on a cost-effectiveness analysis of a screening program in PJS, based on EUS and ERCP for molecular analysis of pancreatic juice. According to this review, patients with suspicious findings would be offered CT, all others should repeat screening 1-3 years later, based on risk stratification detrmined by molecular tests. With this approach over a 35-year period of annual EUS, 3780 screens would be carried out and only those with morphological changes found on EUS are offered CT and ERCP.

This model can give an estimate of costs of about $372708 per life saved. This cost could be further reduced to $297000 per life saved by molecular analysis of pancreatic juice. In this case, in fact, most individuals would only be screened every 3 years thanks to more accurate risk stratification.

FUTURE PERSPECTIVES

In the near future, the development of EUS technology should help us to screen HRIs.

Contrast-enhanced harmonic endoscopic ultrasonography (CH-EUS) visualizes parenchymal perfusion in the pancreas without Doppler-related artifacts[140,141]. It could play a central role associated to EUS-FNA when the latter gives a negative finding in a suspected lesion. Two recent studies[141,142] showed a higher sensitivity of CH-EUS compared to EUS-FNA for the identification of pancreatic carcinoma. Most of false-negative EUS-FNAs resulted to have a hypoenhancement on CH-EUS examination. Moreover, Kitano *et al*[142] found that CH-EUS when combined with EUS-FNA is able to increase the sensitivity from 92.2% to 100% and is superior to MDCT in diagnosing small (< 2 cm) carcinomas, identifying 9 tumours missed by MDCT. Fusaroli *et al*[143] also reported that CH-EUS allowed the detection of small lesions in patients with uncertain EUS findings because of chronic pancreatitis. In addition, CH-EUS allows to focus on the lesion target for EUS-FNA.

Diagnostic accuracy of EUS-FNA will be also enhanced by the detection of DNA abnormalities as k-ras point mutations and microsatellite losses[144,145] or novel protein markers such as mesothelin[146,147] and prostate stem cell antigen[147]. Their detection in EUS-FNA specimens may provide confirmation of the presence or absence of malignancy and should negate the need for further testing.

Characterization of pancreatic cysts has become essential for definitive surgical treatment or ongoing surveillance. However, current diagnostic methods (cross-sectional imaging, EUS, and fluid analysis including cytology, fluid characteristics, chemistry, and tumor markers) do not allow an accurate differentiation between the various types of cysts[148,149]. A novel needle-based confo-

cal laser endomicroscopy (nCLE) miniprobe that can be passed through a 19-G EUS-FNA needle enables real-time imaging with microscopic detail. A pilot study[150] suggests that nCLE can detect mucinous pancreatic neoplasms with excellent specificity and PPV (100% for both of them) but a low sensitivity and NPV (59% and 50%, respectively) with an overall complication rate of 9%.

Finally, computer-aided diagnostic techniques, yet used in some screening programs[151,152], could be added to standard EUS images for the differentiation of pancreatic carcinoma from chronic pancreatitis[151,153]. With digital image processing and computer-aided EUS image differentiation technologies, physicians could use the computer output as a "second opinion" and make the final decisions as reported by the high diagnostic accuracy (98%) of a recent study[154].

CONCLUSION

These data demonstrate that screening with EUS, preferably associated with MRCP, as reported by International Cancer of the Pancreas Screening summit (83.7% agree for EUS and 73.5% agree for MRI/MRCP)[96] is feasible and can detect curable pancreatic neoplasms in correctly identified asymptomatic at-risk patients. In particular, as reported by Ludwig *et al*[155], EUS could be subsequent to an MRCP as initial imaging. This approach should reduce the number of false positives (patients with abnormal MRCPs who on EUS had no appreciable lesion) avoiding unnecessary surgery. The two modalities may complement each other. In fact, MRI/MRCP, in contrast with EUS, is able to image the entire abdomen and pelvis, an useful feature for patients at risk for multi-organ cancer, but has a low sensitivity in detecting PanIN lesions and small (< 1 cm) pancreatic cancer, even if recently there has been the development of 3T MRI scanners able to detects small tumors in asymptomatic patients through indirect signs (black and white sign) and cystic lesions ≥ 3 mm[99,156]. MRCP is superior to EUS in delineating lesions involving the pancreatic ductal system[97,98] even if a recent study[157] has shown similar results between three dimensional CEUS and MRI in evaluating IPMNs smaller than 1 cm. Nevertheless EUS can image mural nodules associated with increased risk of malignancy.

It is also strongly suggested that surveillance programs should be performed by a center with experience in the specific pathology within the context of peer reviewed protocols to reduce interobserver disagreement[100].

Indeed, EUS is an operator-dependent technique that requires considerable skills and training in EUS is essential to gain experience to reliably examine the pancreas. The intensity and length of training, the requisite curriculum and the minimum number of procedures required to ensure competency are not well-defined[158].

Some experts recommend a minimum of 75 pancreatobiliary procedures and 25 cases of pancreatic FNA[159], others suggest a minimum of 30 supervised EUS-FNA

on pancreatic lesions[160] while someones believe that the majority of trainees will require double the number of proposed procedures to achieve competency in EUS[161,162].

An extensive use of CT or ERCP should be avoided in screening programs that require repeated exams in healthy individuals who have only a statistical risk of cancer.

However, a number of questions remain to be answered. What are the significance and natural history of EUS-detected chronic pancreatitis-like abnormalities? What is the clinical significance of PanIN with moderate dysplasia? Should it always be treated with pancreatectomy? How to manage the IPMN-like cystic lesions frequently found in HRIs? Should be offered surgery or a wait-and-see policy can be adopted?

As the resolution of imaging improves and as our knowledge of precursor lesions grows, we believe that these questions will be answered in the future.

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Pathological features and diagnosis of intraductal papillary mucinous neoplasm of the pancreas

Víctor M Castellano-Megías, Carolina Ibarrola-de Andrés, Guadalupe López-Alonso, Francisco Colina-Ruizdelgado

Víctor M Castellano-Megías, Department of Pathology, Hospital Universitario de Fuenlabrada, 28942 Fuenlabrada, Madrid, Spain

Carolina Ibarrola-de Andrés, Guadalupe López-Alonso, Francisco Colina-Ruizdelgado, Department of Pathology, Hospital Universitario "12 de Octubre", 28942 Fuenlabrada, Madrid, Spain

Author contributions: Castellano-Megías VM, Ibarrola-de Andrés C, López-Alonso G and Colina-Ruizdelgado F contributed equally to this work.

Correspondence to: Dr. Víctor Manuel Castellano-Megías, Department of Pathology, Hospital Universitario de Fuenlabrada, Camino del Molino, 2, 28942 Fuenlabrada, Madrid, Spain. victormanuel.castellano@salud.madrid.org

Telephone: +34-916-006331 Fax: +34-913-908462

Abstract

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a noninvasive epithelial neoplasm of mucin-producing cells arising in the main duct (MD) and/or branch ducts (BD) of the pancreas. Involved ducts are dilated and filled with neoplastic papillae and mucus in variable intensity. IPMN lacks ovarian-type stroma, unlike mucinous cystic neoplasm, and is defined as a grossly visible entity ($\geqslant$ 5 mm), unlike pancreatic intraepithelial neoplasm. With the use of high-resolution imaging techniques, very small IPMNs are increasingly being identified. Most IPMNs are solitary and located in the pancreatic head, although 20%-40% are multifocal. Macroscopic classification in MD type, BD type and mixed or combined type reflects biological differences with important prognostic and preoperative clinical management implications. Based on cytoarchitectural atypia, IPMN is classified into low-grade, intermediate-grade and high-grade dysplasia. Based on histological features and mucin (MUC) immunophenotype, IPMNs are classified into gastric, intestinal, pancreatobiliary and oncocytic types. These different phenotypes can be observed together, with the IPMN classified according to the predominant type. Two pathways have been suggested: gastric phenotype corresponds to less aggressive uncommitted cells (MUC1 -, MUC2 -, MUC5AC +, MUC6 +) with the capacity to evolve to intestinal phenotype (intestinal pathway) (MUC1 -, MUC2 +, MUC5AC +, MUC6 - or weak +) or pancreatobiliary /oncocytic phenotypes (pyloropancreatic pathway) (MUC1 +, MUC 2-, MUC5AC +, MUC 6 +) becoming more aggressive. Prognosis of IPMN is excellent but critically worsens when invasive carcinoma arises (about 40% of IPMNs), except in some cases of minimal invasion. The clinical challenge is to establish which IPMNs should be removed because of their higher risk of developing invasive cancer. Once resected, they must be extensively sampled or, much better, submitted in its entirety for microscopic study to completely rule out associated invasive carcinoma.

Core tip: The authors review the main pathological features of intraductal papillary mucinous neoplasm (IPMN) of the pancreas, including diagnostic criteria and relevance of macroscopic (*i.e.*, main duct, branch duct and mixed or combined) and microscopic (*i.e.*, gastric, intestinal, pancreatobiliary and oncocytic) IPMN classification. Different pathways, mucin immunophenotypes and invasive carcinoma related to IPMN are addressed. Differential diagnosis with pancreatic intraepithelial neoplasm, mucinous cystic neoplasm and other mucinous and non-mucinous pancreatic cystic lesions are

also included.

DEFINITIONS AND INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas is a grossly visible, noninvasive epithelial neoplasm of mucin producing cells arising in the main pancreatic duct or its branches. Intraductal growth of neoplastic cells usually forms papillae in a variable extension, although it can rarely be completely flat. Involved ducts are dilated and filled with mucus in variable intensity[1-4]. The mucus produced by the IPMN can protrude through the duodenal papilla and this sign, so-called "fish-eye ampulla", is virtually diagnostic, although it has been observed in only about 25% of cases[5,6]. IPMN lacks ovarian-type stroma, unlike mucinous cystic neoplasm (MCN) of the pancreas[7]. IPMN is defined as a grossly visible entity, unlike pancreatic intraepithelial neoplasm (PanIN), which is defined as a microscopic lesion[8].

IPMN was previously reported under a variety of terms (mucinous producing cancer[9], ductectatic-type mucinous cystadenoma and cystadenocarcinoma[10], diffuse villous adenoma[11] and intraductal papillary neoplasm of the pancreas[12]) that referred to some of the main features of these lesions. Currently, use of these terms is discouraged. Intraepithelial papillary lesions morphologically analogous to IPMNs develop in the biliary tree, including bile ducts[13], gallbladder[14] and ampullary region[15], and are also reported under a variety of terms.

Most IPMNs are diagnosed between 60 and 70 years of age. There is a slightly higher prevalence in men than women[16]. IPMNs are mostly located in the pancreatic head (70%). About 20% are placed in the body-tail and about 5%-10% show diffuse involvement of the gland. Most are solitary lesions but 20%-40% are multifocal[1,17]. IPMNs can reach a large size before diagnosis because of the slow and indolent growth. However, with the use of high-resolution imaging techniques, very small incidental pancreatic cysts, including IPMNs, are increasingly being identified[18,19]. In the Laffan *et al*[19] series, radiological incidental pancreatic cysts were detected with a mean size of 8.9 mm (range 2-38 mm) in 2.6% of adults without known pancreatic disease. It has been suggested that most of them are IPMNs originating from the small branch ducts but pathological data are missing[20]. On the other side, older surgical data, in which IPMN represents 20% of all pancreatic cysts, probably underestimate its prevalence[1].

IPMNs may exhibit different degrees of dysplasia in the epithelium but even those with high-grade dysplasia
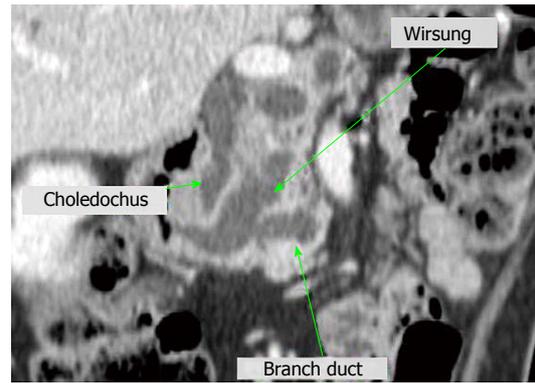
Figure 1 Computerized tomography scan demonstrating massive dilatation of the main pancreatic duct and its branches. Obstructed bile duct is also dilated.

or carcinoma in situ have a very good prognosis after resection[21-24]. However, about 40% of IPMNs show invasive pancreatic carcinoma at diagnosis[18]. The prognosis of these patients critically worsens when invasive carcinoma arises, although in the case of minimal invasion, the prognosis is not as severe[23,25]. The clinical challenge is to establish which IPMNs can be managed by clinical and radiological follow-up without requiring surgical excision and which should be removed because they are likely to develop invasive cancer. Progress has been made in the preoperative assessment of the risk of malignancy of pancreatic cystic lesions. Currently, there is an international consensus for the preoperative management of these patients based on clinical and radiological criteria, published in 2006 (the so called Sendai criteria) and updated in 2012 (the Fufuoka guidelines)[7,18]. Nevertheless, the preoperative diagnostic accuracy of pancreatic cystic neoplasms is still far from optimal[26,27].

MACROSCOPIC PATHOLOGY

IPMN appears as a dilatation of the main duct or as one or more cysts communicated with the excretory duct system. IPMNs are cystic lesions so the observation of any solid nodule should be suspected of associated invasive carcinoma. However, it should be noted that the invasive carcinoma, especially if small, may be overlooked macroscopically.

DEFINING SIZE OF IPMN

IPMN is defined as a grossly visible lesion and, mostly based on radiological criteria, it is typically considered to be 1 cm or more in size[1,7] (Figure 1). More recently, the Fukuoka guidelines have proposed to reduce the minimum size for radiological diagnosis of IPMN to 5 mm, which increases diagnostic sensitivity without losing specificity. According to this consensus, pancreatic cysts of > 5 mm in diameter that communicate with the main pancreatic duct, especially if there is no pancreatitis, and/or diffuse dilation of the main pancreatic duct of > 5 mm without other causes of obstruction are sufficient

Main duct type

Branch duct type
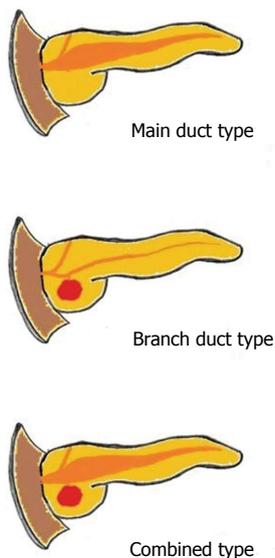
Combined type

Figure 2 Scheme of macroscopic classification of intraductal papillary mucinous neoplasm.

radiological criteria for IPMN[18].

Main duct, branch duct and mixed or combined types

According to their main location, IPMNs are classified into main duct (MD) type (16% to 36%), branch duct (BD) type (40% to 65%) and mixed or combined type (15% to 23%)[21,23,28,29] (Figure 2). This classification reflects biological differences with important prognostic implications[23,30]. Most MD type and combined type IPMNs exhibit malignancy (*i.e.*, high-grade dysplasia or carcinoma), with about 45% having an associated invasive carcinoma[18,22,31]. In contrast, most BD type show low-grade dysplasia and only about 15% are associated with invasive carcinoma[18]. The natural history of BD type under 30 mm in size and without mural nodules is particularly favorable[5,32]. Currently, this macroscopic classification has a substantial practical impact on the preoperative clinical management based on imaging findings[7,18].

MD type (Figure 3A) is essentially located in the main pancreatic duct[11,18]. At external examination, the pancreas may be thickened in the affected area. After opening, this type typically shows dilatation of the main duct with irregular outline and the lumen is filled by mucus and villous or papillary projections. The rest of the pancreas often shows the appearance of obstructive chronic pancreatitis due to pancreatic duct obstruction[17]. Most of the MD type are located in the pancreatic head but one third of them are in the body and tail, and almost 5% in the entire main pancreatic conduct (diffuse MD type)[22]. Eventually, some cases of MD type are multifocal. However, a particular lesion may be macroscopically from a focal type but microscopically exhibit multifocal or diffuse extension throughout the duct.

BD type (Figure 3B) is located predominantly in secondary branches of the pancreatic ductal system[1,18]. Typically, the affected duct has the appearance of a mucus-filled cyst. As there is no main pancreatic duct

obstruction, the remaining pancreas may have normal appearance[17,33]. Most of the IPMN of BD type occurs in the pancreatic head and very commonly in the uncinate process. About 25%-40% are multifocal[18,22].

The mixed or combined type of IPMN (Figure 3C) primarily affects both the main pancreatic duct and secondary branches[1,18]. Hypothetically, combined type of IPMN might result from the progression of MD or BD types or it could be a distinct disease. Clinical and biological characteristics are similar to those of the MD type, so it is thought that combined IPMN is most likely an extension of the MD type to the branch ducts[22,30].

Fistula formation and other additional macroscopic features of IPMNs

Uncommonly, the neoplastic papillae extends out of the ampulla and onto the surface of the periampullar duodenum or into the distal common bile duct[17].

Also infrequently, IPMN can develop a fistula to neighboring organs, among them the duodenum, stomach, choledochus, colon and small intestine. The fistula may be related to benign IPMNs (*i.e.*, low or moderate degree of dysplasia), malignant IPMNs (*i.e.*, high-grade dysplasia) or invasive carcinoma associated with IPMN (often a colloid carcinoma)[34]. Two scenarios can be observed in the pathogenesis of these fistulas: (1) mechanical penetration due to excessive pressure in the mucin filled ducts, in addition to inflammatory stimulation or autodigestion of enzyme rich fluids; and (2) direct invasion, *i.e.*, with presence of invasion of the tissue around the fistula[34,35]. Mechanical penetration can occur regardless of the presence of malignant cells at the surface of the fistula (without direct tissue invasion by carcinoma). In conclusion, the presence of fistulas in IPMN does not necessarily mean malignancy and should not be confused with invasive carcinoma.

In rare cases, IPMN has been described as causing *pseudomyxoma peritonei*[36], for instance by associated acute pancreatitis with fistula formation to the abdominal cavity[37] or after intraoperative manipulation of the pancreas[22].

Extensive *pancreatic calcification* has rarely been described in patients with IPMN. This obstructive calcifying pancreatitis, presumed to be caused by the IPMN, may lead to preoperative diagnostic confusion and delay in the diagnosis of the papillary neoplasia[38,39].

MICROSCOPIC PATHOLOGY

Histologically, IPMN is a heterogeneous group of lesions with different degrees of dysplasia and different cellular phenotypes. The underlying stroma shows a conventional fibrous tissue, which by definition can not be of ovarian type, as seen in MCN[1].

Grades of dysplasia

Based on the degree of cytological atypia and abnormal crowding of the epithelium, IPMN is classified into three categories: IPMN with low-grade dysplasia, IPMN with intermediate-grade dysplasia and IPMN with high-grade

Figure 3 Macroscopic classification of intraductal papillary mucinous neoplasm. A: Main duct type; B: Branch duct type; C: Combined type.
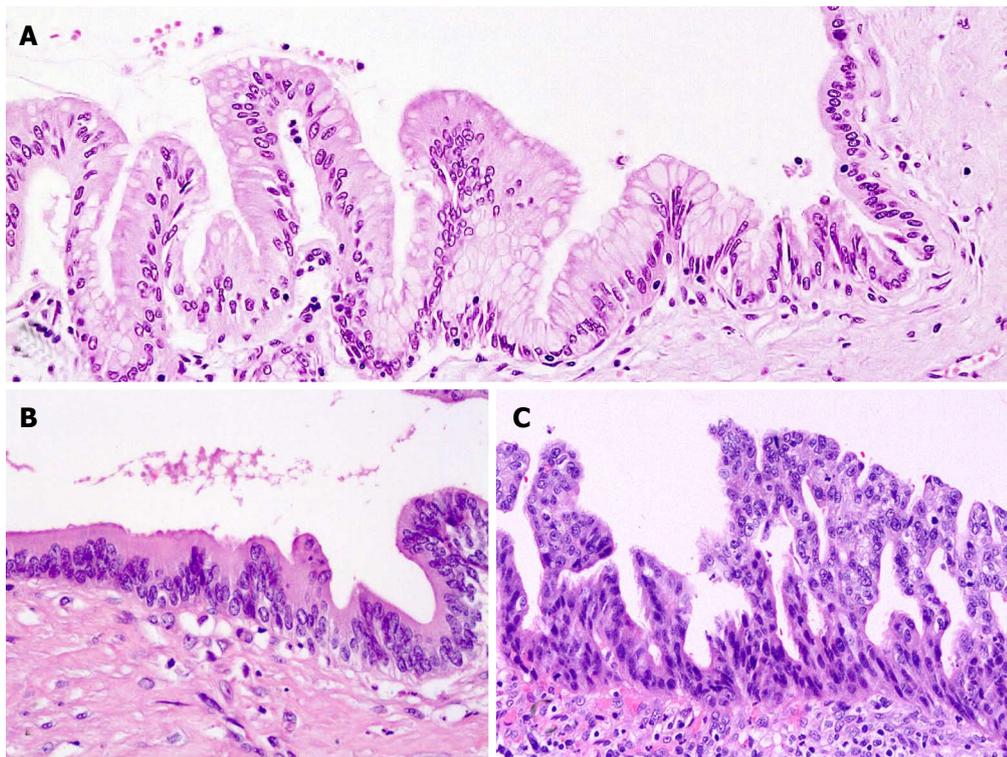

Figure 4 Different degrees of dysplasia. A: Low-grade (gastric IPMN). See transition with non dysplastic normal duct epithelium (right side); B: Intermediate-grade (intestinal IPMN); C: High-grade (pancreatobiliary IPMN). IPMN: Intraductal papillary mucinous neoplasm.

dysplasia (Figure 4). This nomenclature, currently adopted by the WHO system, replaces the terms of adenoma (low grade), borderline (intermediate grade) and carcinoma *in situ* (high grade dysplasia)[1,7]. Low-grade dysplasia is characterized by a uniform monolayer of columnar cells with basal nuclei showing no or minimal atypia. In the intermediate-grade of dysplasia, nuclear atypia is higher, with nuclear pleomorphism, nuclear enlargement and pseudostratification. In high-grade dysplasia, there is marked cytological atypia and complex architecture with cribriform groups and budding of neoplastic cells into the lumen[4,17]. It is common to observe different grades of dysplasia within a given lesion, which suggests the development of dysplasia from a lower to a higher grade. The distinction of dysplasia grade is important, with associated invasive carcinoma commonly immersed in areas of high-grade dysplasia[40,41]. In each individual case, the lesion should be classified according to the highest grade

of dysplasia observed[1].

Mucins and microscopic IPMN phenotypes

On the basis of the cytoarchitectural features and immunophenotype, IPMNs are classified into four histopathological types: gastric, intestinal, pancreatobiliary and oncocytic IPMNs[1], accounting for 49%-63%, 18%-36%, 7%-18% and 1%-8% of total cases in two large series[3,42] (Table 1). This classification is not only descriptive but also indicative of different pathways of differentiation and progression to invasive carcinoma[23,43-48]. The above nomenclature prevails over other terms proposed for these lesions[3]. The so called villous dark cell, papillary clear cell and compact cell types respectively correspond to intestinal, gastric and oncocytic cell types[49,50], whereas null cell type corresponds to gastric cell type[51].

Core proteins for mucins (MUCs) can be detected by immunohistochemistry. The mucin expression profile by
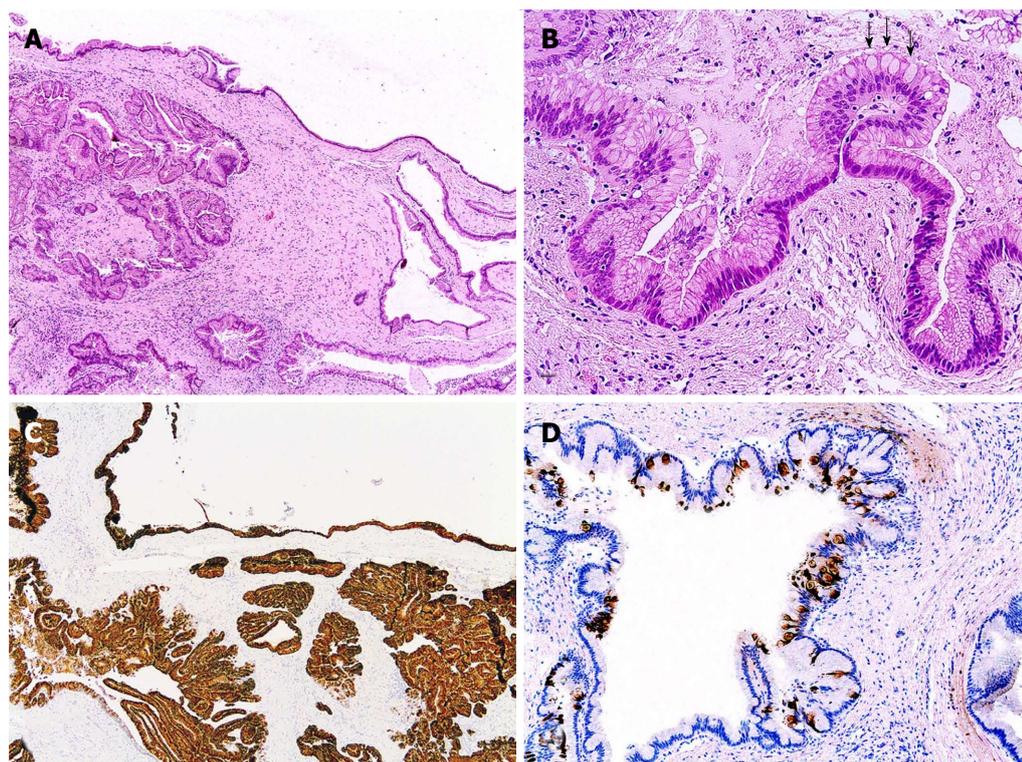
Figure 5 Gastric intraductal papillary mucinous neoplasm. A: The neoplasm involves branch ducts with a multicystic appearance; B: Columnar cells with basal nucleus and apical mucin. Notice the scattered goblet cells (arrows); C: Immunohistochemical MUC5AC expression (colored brown by diaminobenzidine); D: MUC2 highlighting the goblet cells. MUC: Mucin.

Table 1 Mucin immunoprofile in intraductal papillary mucinous neoplasm

Morphological type	MUC1	MUC2	MUC5AC	MUC6
Gastric	-	-[1]	+	+
Intestinal	-	+	+	± weak
Pancreatobiliary	+	-	+	±
Oncocytic	+	-[1]	±[1]	+

[1]Scattered positive goblet cells can be present. MUC: Mucin.

the IPMN cells is a major contributor to their phenotypic classification. Mucins are high molecular weight glycoproteins produced by different types of epithelial cells. Some mucins are normally located in the cell membrane, like MUC1 (also called mammary-type mucin or pan-epithelial membrane mucin), whereas others are normally secretory products, including MUC2 (intestinal type gel forming mucin), MUC5AC (gastric surface mucous epithelial mucin) and MUC6 (gastric pyloric glandular mucin)[52,53].

In normal pancreatic tissue, there is MUC1 expression (limited to centroacinar cells, intercalated and intralobular ducts and focally in the interlobular ducts) and sometimes there is expression of MUC6 (limited to the acini) but MUC2 and MUC5AC are not expressed[50]. In pancreatic neoplasms, MUC1 is considered a marker of aggressiveness, being expressed in some IPMNs, higher-grade cases of PanIN and in conventional (*i.e.*, tubular) ductal adenocarcinoma. On the contrary, MUC2 is considered a marker of a more indolent phenotype, being

expressed in some IPMNs and in colloid carcinoma[43].

Gastric type IPMN is frequently observed in BD type. The great majority of gastric IPMNs exhibit only low-grade dysplasia and association with invasive carcinoma is uncommon. It has been observed that, when developing, invasive carcinomas are conventional type with more aggressive characteristics and with a poorer prognosis than those arising from intestinal or pancreatobiliary IPMNs[23,16]. Because the gastric type IPMNs associated with these invasive carcinomas is usually benign (*i.e.*, with only low or intermediate grade of dysplasia), it has been questioned whether in these cases the gastric type IPMN represents the invasive carcinoma or whether it is just its background or coexisting benign IPMN[42]. Gastric type IPMN consists of columnar cells with basal nuclei and abundant apical cytoplasmic mucin, resembling the foveolar gastric epithelium (it is also called gastric foveolar type IPMN). These lesions are often mainly flat or with low papillary pattern, consisting in thick finger-like papillae[3]. Immunoprofile consists of diffuse expression of MUC5AC and MUC6 without expression of MUC1 and MUC2, although scattered MUC2 positive goblet cells can be present into the lesion[16,44,48,50]. Gastric mucins may show a distribution which mimics mucins found in the gastric mucosa, namely, increased expression of MUC5AC in the superficial or papillary areas, simultaneously of MUC6 located in the basal areas[50] (Figure 5).

Intestinal type IPMNs mimics villous adenomas of the colon. They form elongated papillae of columnar cells with enlarged cigar-like nuclei[3]. Diffuse expression
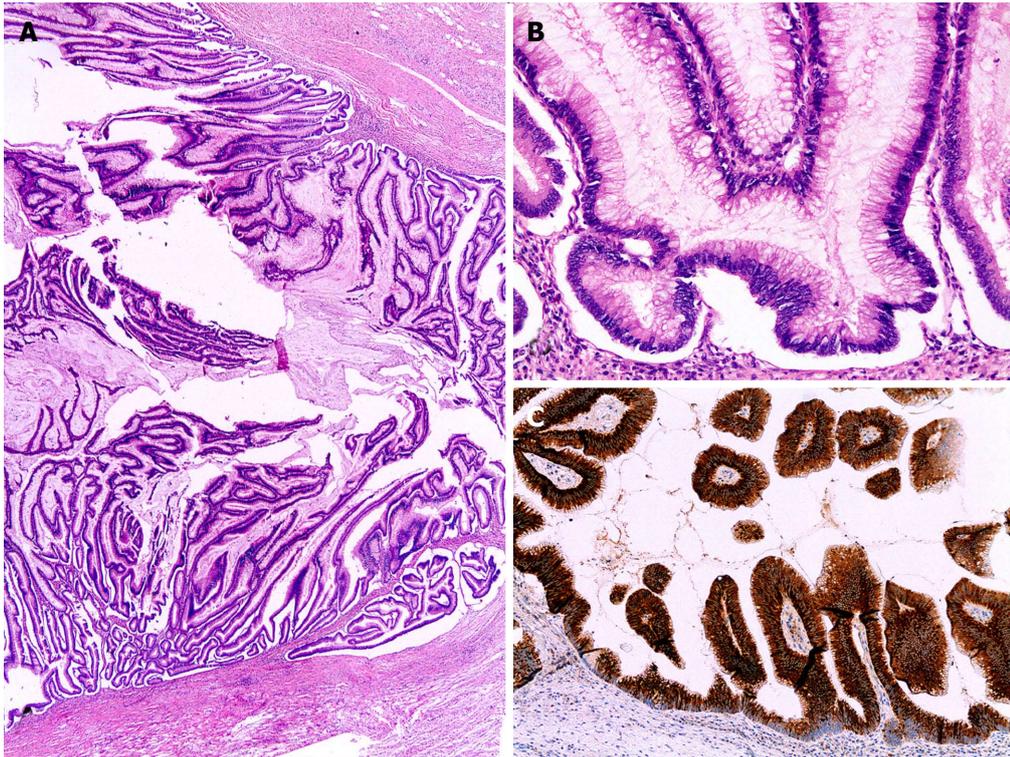
Figure 6 Intestinal intraductal papillary mucinous neoplasm. A: Main duct distended by long papillae; B: Projections of columnar cells with pseudostratified nuclei; C: Immunohistochemical MUC2 expression. MUC: Mucin.

of MUC2 and MUC5AC, weak or negative expression of MUC6 and negativity for MUC1 is the mucin immunoprofile of this type[16,50,48]. In addition, intestinal IPMN exhibits diffuse expression for CDX2, a transcriptional factor related to intestinal differentiation that, like MUC2, has tumor suppressor activity[54]. The intestinal type IPMN frequently exhibits an intermediate or high-grade of dysplasia. It occurs more frequently in the main duct and, when associated with invasive carcinoma, this is often a colloid adenocarcinoma[23,51]. In the absence of invasive carcinoma, intestinal IPMN seems to have a greater potential for long-term recurrence than non-invasive IPMN of other types. Recurrence in the remnant pancreas may be due to multifocality not recognized at the time of surgery or to metachronous development[23] (Figure 6).

Pancreatobiliary type IPMNs usually show high-grade dysplasia and is likely to have a strong predisposition to develop invasive carcinoma[23]. Associated invasive carcinomas usually are of conventional type[23,51]. Pancreatobiliary type IPMN consists of more cuboidal cells with rounded nuclei, often with prominent nucleoli. The neoplastic cells are organized into thin complex and branching papillae with bridging and cribiform patterns[3]. The neoplastic cells express MUC1 and MUC5AC, sometimes MUC6, and are negative for MUC2[3,48] (Figure 7).

Oncocytic type IPMNs (also known as intraductal oncocytic papillary neoplasm) are characterized by neoplastic cells with abundant granular eosinophilic cytoplasm (due to the presence of numerous mitochondria) and also intracellular mucin[1,55]. Most of them have high-grade dysplasia, with complex thick papillae and crib-

riform structures. The neoplastic cells express MUC1 and MUC6. Expression of MUC5AC is controversial, being constantly present according to some authors but limited to scattered goblet cells according to others. The scattered goblet cells also express MUC2[48,56,57]. Invasive carcinoma associated with oncocytic type IPMN often conserves the oncocytic features[23,42]. An association between the oncocytic type IPMN and minimally invasive carcinoma has been observed[23].

Different pathways in IPMNs

In some cases, different phenotypes can be observed in the same lesion. Each lesion should be classified according to the predominant phenotype, although all the present phenotypes should be recorded[3]. The most common coexistences are gastric with intestinal or gastric with pancreatobiliary type[42]. On the contrary, it is very rare to observe intestinal and pancreatobiliary types together[16]. Oncocytic type has been observed to be associated with gastric and pancreatobiliary types[56]. It has been suggested that the gastric phenotype corresponds to less aggressive uncommitted cells with the capacity to evolve to intestinal phenotype (intestinal pathway) or pancreatobiliary/oncocytic phenotypes (pyloropancreatic pathway) becoming more aggressive[44,48,51]. Gastric foveolar epithelium-like cells (also called null cell type cells) similar to cells of the gastric papillary areas of IPMNs can usually be observed lining the nonpapillary cystic areas of different IPMNs[51]. Pancreatic duct glands are blind-ending outpouches of major ducts with a possible role in epithelial renewal and repair. Epithelium of these glands
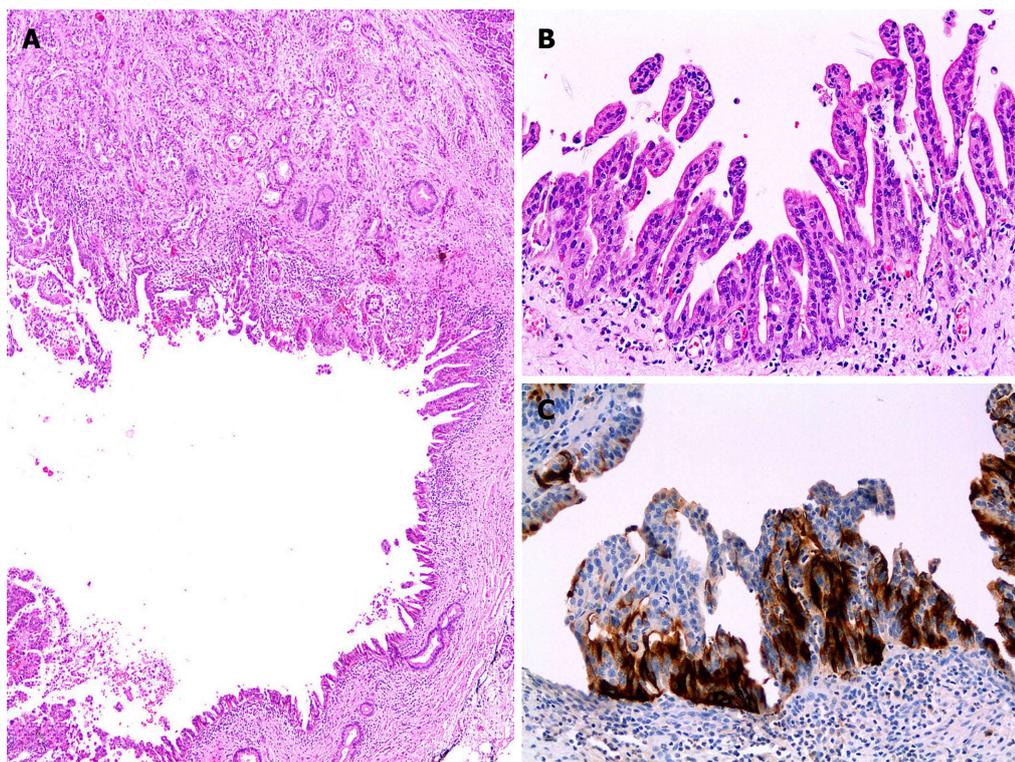
Figure 7 Pancreatobiliary intraductal papillary mucinous neoplasm. A: Intraductal papillary mucinous neoplasm with associated conventional duct carcinoma (upper side); B: Small thin papillae with cuboidal neoplastic epithelium; C: Immunohistochemical MUC1 expression. MUC: Mucin.

is a specialized compartment with production of gastric type mucin (MUC6+) in its normal state. When chronically injured, it becomes hyperplastic and it acquires de novo expression of MUC5AC. It has been speculated that these pancreatic duct glands are a source of gastric mucinous metaplasia and could be the origin of PanIN[58] and IPMNs[6], in addition to its possible role in regeneration and protection of the major ducts.

Intraoperative microscopic assessment of pancreatic margin

Frozen study of pancreatic cut surface during resection of IPMN is accurate to evaluate the completeness of resection. The accuracy of frozen study averages 95%[59]. If invasive carcinoma or high-grade dysplasia is seen in the pancreatic margin, this should be extended. Further resection in cases with lesser degrees of dysplasia in the margin is controversial[18] but may be considered in some cases, depending on the patient's age and the macroscopic type IPMN among other factors[60,61]. Recurrences after resection of non-invasive IPMN with free margin may occur and can be attributed to multifocality[60].

The distinction between IPMN and PanIN may be almost impossible in some cases, although this distinction is not considered crucial in assessing the margin, with the distinction of the degree of dysplasia being most relevant[18]. Those wishing to obtain a pancreatic margin without any degree of dysplasia should be aware that intraoperative differential diagnosis of low-grade dysplasia *vs* non dysplastic epithelium with reactive changes can be impossible to achieve. This should be considered to avoid unnecessary or useless resections. The presence of mucus or duct dilatation at the cut surface does not indicate any additional necessity for resection. De-epithelization of the pancreatic duct margin has been observed to be a prognostic factor for recurrence by some authors and thus they have proposed to do additional resection in cases of eroded epithelium[60].

IPMN AND INVASIVE CARCINOMA

Adenocarcinoma derived from vs concomitant with IPMN

About 40% of IPMNs are associated with invasive pancreatic carcinoma, although the reported risks of malignancy are quite population-dependent and vary considerably (with range between 1.4% and 80.8%)[18,29]. Invasive carcinoma can be uni- or multifocal and occurs most often in MD and combined type than in BD type[18,22]. In patients with IPMN, the distinction must be made between adenocarcinoma derived from IPMN and adenocarcinoma concomitant with IPMN[62]. The first evidently develops from IPMN, while the latter occurs in the pancreas with IPMN but in another location of the organ, therefore without an obvious topological relationship and in the absence of histological transition between the two lesions. Sometimes, the possible relationship between IPMN and invasive carcinoma remains undetermined. In a large series of patients with IPMNs and associated adenocarcinoma, 66%, 17% and 16% corresponded to adenocarcinoma derived from IPMN, concomitant with IPMN, and undetermined, re-
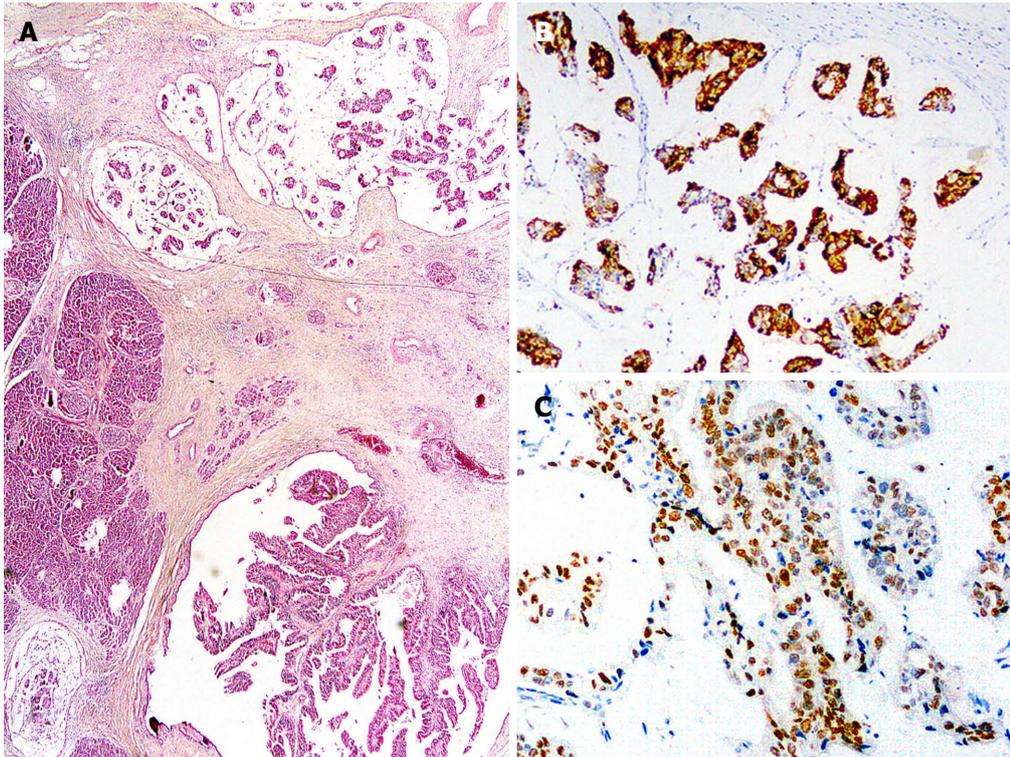
Figure 8 Colloid carcinoma. A: Invasive neoplastic cells floating in pools of mucin (upper side) and associated with intraductal papillary mucinous neoplasm (lower side); B: Immunohistochemical MUC2 expression; C: CDX2 nuclear immunoexpression. MUC: Mucin.

spectively[63]. Among the general population with IPMN, the risk of developing invasive pancreatic carcinoma separately from IPMN was estimated to be 2.8% in a recent cohort analysis[29].

Principal histological types of invasive carcinoma related to IPMN

Most invasive carcinomas related to IPMNs are colloid (mucinous noncystic) carcinomas and conventional (tubular) carcinomas. Mixed colloid-tubular carcinomas or adenocarcinomas with focal colloid features also occur[17,23,24, 29,63-65]. Colloid carcinoma of the pancreas is very commonly associated with IPMN[51]. In fact, some authors argue that it virtually never exists without an associated IPMN (whose detection would depend on the extent of the tumor sampling)[65]. Most IPMNs related to colloid carcinoma are intestinal type[23]. Like intestinal type of IPMN, colloid carcinoma shows diffuse expression of CDX2 and MUC2 (*i.e.*, features of intestinal differentiation)[51] (Figure 8). Prognosis of colloid carcinoma is considered better than conventional ductal carcinoma[18,66]. Conventional invasive adenocarcinoma related to IPMN is most often associated with pancreatobiliary type IPMN (Figure 7A). Both share the more aggressive immunohistochemical profile consisting of MUC1 expression and lack of MUC2 and CDX2 expression[23,51].

Prognosis aspects linked to the existence of invasive carcinoma in IPMN

In general, the prognosis of patients with invasive carcinoma associated with IPMN is better than that of patients with ordinary pancreatic adenocarcinoma (*i.e.*, patients without IPMN), but when matched by disease stage, no prognostic differences appear to exist between the groups, except at an early stage. Best overall prognosis of these patients may lie in their greater frequency of early stage cases and a higher prevalence of colloid carcinomas[21,41,67,68]. A lower frequency of other adverse histological features (*i.e.*, vascular invasion, perineural invasion, involvement of surgical margins and poor tumor differentiation) contribute to better prognosis of IPMN-associated invasive carcinomas[69].

Some authors have observed that invasive carcinoma has a better prognosis if depth invasion is limited[23,25,70-72]. This so called minimally invasive carcinoma has been defined as tumor with slight invasion beyond the pancreatic duct wall[23,70,71] or as carcinoma with infiltration depth up to 5 mm[25,72]. Fukuoka guidelines recommend avoiding use of the term "minimally invasive" because of its variable definition. Instead, it is proposed to substage the category T1 into T1a if carcinoma infiltrates up to 0.5 cm, T1b if > 0.5 cm up to 1 cm, and T1c if infiltrates between 1 and 2 cm[18]. Minimal invasion is more frequently observed in intestinal and oncocytic types of IPMNs than in gastric and pancreatobiliary types[23,72]. Frequently, minimally invasive carcinoma related to IPMN is colloid type[63].

Some patients with resected IPMN without associated invasive carcinoma subsequently develop local invasive carcinoma or metastatic lesions. Multifocal disease with synchronous or metachronous development of tumor in the remnant unresected pancreas may explain the origin
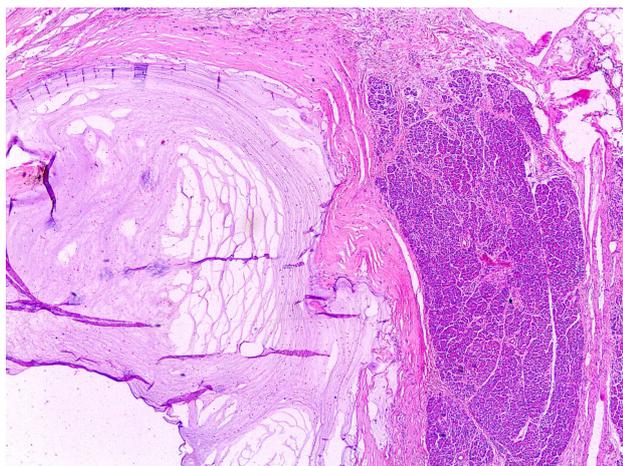
Figure 9 Pseudoinvasion. Mucin spillage dissecting into the pancreatic stroma without neoplastic cells.

of some of these recurrences[24]. In other cases, it is possible that a preexisting small focus of invasive carcinoma passed unnoticed in the pathological study. Because of its critical prognostic significance, a major objective for the pathologist is to rule out the presence of invasion. Initial sampling of the surgical specimen should include all nodular areas because of its higher suspicion of malignancy. As invasive carcinoma may be overlooked by gross examination (especially if it is of small size), the tumor must be extensively sampled or, much better, submitted in its entirety for microscopic study. Measuring the size of invasive carcinoma irrespective of the size of IPMN is required for appropriate staging. If multiple invasive foci exist, they must be measured separately, highlighting the size of the largest focus.

Invasion vs pseudoinvasion

There is usually little difficulty in recognizing invasive carcinoma associated with IPMN when tumor cells are observed penetrating the tissue with a classic infiltrative growth pattern. However, like in other mucin-secreting tumors, IPMNs can exhibit tumor growth by duct expansion (expansive growth) as well as mucous rupture or mucin spillage into the stroma, whose interpretation is controversial[25,73]. Lakes of mucin in the stroma may correspond to colloid carcinoma but also may be due to rupture of a mucus filled duct, presumably by the high intraluminal pressure produced by the mucus itself. IPMN desquamated cells could be transported to the stroma by the extruded mucin, completely simulating colloid carcinoma. Acellular mucin extruded into stroma is not considered invasive cancer (Figure 9). In contrast, mucin spillage containing neoplastic cells is better considered invasive carcinoma[1,17]. On another issue, IPMNs should not be confused with the rarest pancreatic adenocarcinomas with cystic papillary pattern, consisting of large caliber malignant glands with intraluminal papillary structures and pools of intraluminal mucin that mimic noninvasive cystic neoplasms. Elastin stains are very helpful for distinction: unlike normal pancreatic ducts and

ducts with IPMN that typically are surrounded by a layer of elastin fibers, there are no elastin fibers around these large invasive malignant glands[74].

DIFFERENTIAL DIAGNOSIS

Other cystic and/or papillary lesions

A major preoperative (*i.e.*, mainly clinical and radiological) differential diagnosis of IPMNs includes neoplastic and non-neoplastic pancreatic cysts: serous cytadenoma (in particular the oligocystic or macrocystic variant)[75], MCNs, solid pseudopapillary neoplasm[76], retention cyst[4], pseudocyst and other less common or clinically relevant entities[20]. In addition, usually solid pancreatic lesions that occasionally are dominated by a cystic, papillary or papillocystic pattern must be considered in this differential diagnosis: acinar cell carcinoma[77], pancreatic endocrine tumors[78] and pancreatic duct adenocarcinoma[74]. In surgical specimens, histological findings usually allow solving the above cited differential diagnosis, sometimes with the assistance of immunohistochemistry. In preoperative management, cyst fluid or pancreatic juice cytology can increase clinical and radiological accuracy diagnoses in pancreatic cysts regarding the distinction between mucinous and non mucinous lineage and malignancy identification. Mucinous cyst cytology cannot accurately discriminate between IPMN and MCN and, although cytology shows high specificity for detecting malignancy, sensitivity is low[79]. Sensitivity for malignancy detection increases if cases with cytological diagnosis of high grade atypia are included, but this reduces the specificity[80,81]. Carcinoembryonic antigen (CEA) pancreatic cyst fluid level may contribute to distinction between mucinous (high CEA levels) and non mucinous (low or no CEA levels), but does not differentiate between benign and malignant. Some authors warn about IPMN dissemination after puncture because of the potential risk of leakage of cyst content. Currently, Fukouka guidelines consider cytological study of mucinous-like cystic lesions in general limited to research, except in centers with expertise in endoscopic ultrasound - fine needle aspiration (EUS-FNA) and cytological interpretation where cytological analysis is recommended for the evaluation of small BD-IPMNs without worrisome features[18]. EUS-FNA with cyst fluid CEA determination may also be required for the differentiation between BD-IPMN and oligocystic serous cystic neoplasm[18,75].

MCN and other mucinous cysts

Focusing on the mucinous category, accurate distinction is not always possible between various mucinous cystic lesions. In a large series of resected mucin-producing neoplasms of the pancreas, 6% of mucinous cystic lesions were undetermined[22]. BD type of IPMN, especially when largely flat, can be confused with MCN if the topological branch ducts relationship is not clear. In addition, although MCN lacks a connection to the duct system, it rarely can fistulize into the ducts and very rarely exhibits intracystic papillary-like growth that may be confused
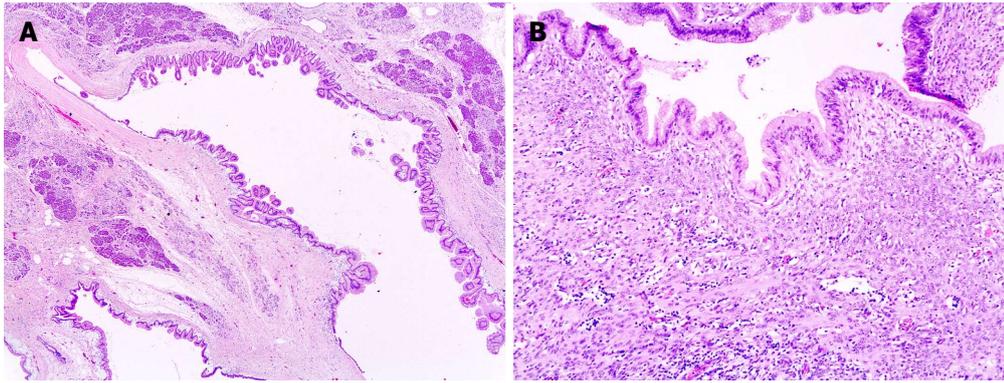
Figure 10 Mucinous cystic neoplasm. A: An example with papillary projections and surrounded by a thick collagenized band; B: Demonstration of ovarian-type stroma, at least focally, leads to diagnosis.

with papillary structures of MD-IPMN[82] (Figure 10A). Ovarian-type stroma in MCN facilitates this differential diagnosis and accentuates the clinical distinction of MCN *vs* IPMN[7] (Figure 10B). MCN occurs in patients usually younger than BD type of IPMN (44.5 years *vs* 66 years in a large series[22]) and it is almost always a single lesion located in the pancreatic body/tail in women, whereas this type of IPMN occurs more commonly in the pancreatic head, can be single or multifocal, and occurs slightly more often in men[7,22]. Cystic mucin-producing pancreatic neoplasms without either IPMN histological features or ovarian-type stroma are better termed indeterminate mucin-producing neoplasms[7]. If such indeterminate cystic lesions exhibit simple mucinous epithelium without cytological atypia, they still are considered neoplasms by some authors[64]. Alternatively, they are termed non neoplastic (or non dysplastic) cystic mucinous lesions by others[83], although it is unclear whether they could represent the earliest manifestation of mucinous neoplasms. Retention cysts should also be considered. They happen because of pancreatic duct obstruction. They usually are unilocular and lined by normal or flattened ductal epithelium without atypia, but sometimes they are described with slight papillary or mucinous change[4]. Therefore, there are no specific limits for the distinction between retention cyst, non neoplastic mucinous cyst and some neoplastic mucinous cysts.

PanIN

PanIN is the other main premalignant lesion of the pancreas besides IPMN. Lesions of PanIN can be flat, micropapillary or papillary, but unlike IPMN which is macroscopically visible, PanIN is defined as a microscopic entity[8]. Although PanIN lesions typically arise in the smaller ducts, it may involve large ducts. In addition, IPMN often extends from larger ducts to smaller pancreatic ducts[4]. The histological distinction between IPMN and PanIN is not always possible. The main issue concerns BD gastric type of IPMN because of its peripheral location and more similar cytohistological appearance and immunohistochemical profile (MUC2 negative and MUC5AC positive)[43,61]. It has generally been assumed that

PanIN measures less than 0.5 cm and IPMN over 1 cm. It has been suggested to use a descriptive diagnosis, such as intraductal proliferative lesion of undetermined type, for an especially gray area of 0.5-1 cm featureless diameter[61].

Intraductal tubulopapillary neoplasm

Intraductal tubulopapillary neoplasm (ITPN) is a rare lesion characterized by a more solid intraductal growth without visible mucin secretions and with less cystic aspect than IPMN. Histologically, ITPN is characterized by a complex proliferation of tubules and variable extension of papillary architecture. Neoplastic cells show scant cytoplasmic mucin and uniform high grade dysplasia. Solid areas and necrotic foci are frequently seen. Associated invasive carcinoma is frequently scarce and observed in about 40% of cases[1,84]. ITPN is considered within the spectrum of IPMNs by some authors, although it is regarded as a separate entity by the current WHO system[16].

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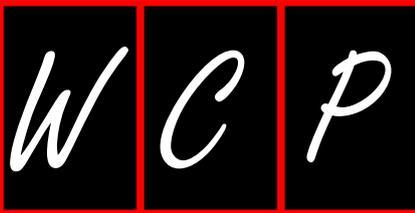
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Radiology of pancreatic neoplasms: An update

Luis Gijón de la Santa, José Antonio Pérez Retortillo, Ainhoa Camarero Miguel, Lea Marie Klein

Luis Gijón de la Santa, José Antonio Pérez Retortillo, Ainhoa Camarero Miguel, Lea Marie Klein, Department of Radiology, Guadalajara University Hospital, University of Alcalá, 19002 Guadalajara, Spain

Author contributions: Gijón de la Santa L designed research; Klein LM is a native speaker of English and revised the manuscript; Gijón de la Santa L, Pérez Retortillo JA were responsible for literature search and picture selection; Camarero Miguel A, Klein LM contributed to the literature review; all authors have contributed to the performed research and wrote the paper.

Correspondence to: Luis Gijón de la Santa, MD, Department of Radiology, Guadalajara University Hospital, University of Alcalá, Donantes de Sangre st, 19002 Guadalajara, Spain. lgijon@sescam.jccm.es

Telephone: +34-949-209200 Fax: +34-949-209218
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Abstract

Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. We describe the imaging features of pancreatic malignancies and their benign mimics. Accurate detection and staging are essential for ensuring appropriate selection of patients who will benefit from surgery and for preventing unnecessary surgeries in patients with unresectable disease. Ultrasound, multidetector computed tomography with multiplanar reconstruction and magnetic resonance imaging can help to do a correct diagnosis. Radiologists should be aware of the wide variety of anatomic variants and pathologic conditions that may mimic pancreatic neoplasms. The knowledge of the most important characteristic key findings may facilitate the right diagnosis.

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Key words: Pancreas; cancer; Radiology; Computed tomography; Magnetic resonance imaging; Surgery; Pancreatic neoplasms

Core tip: Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. We describe and illustrate the imaging features and key findings of pancreatic malignancies and their mimics. The knowledge of radiologic findings is relevant to do an accurate diagnosis that allows a proper management and should be known not only for radiologists but by physicians that comprise multidisciplinary teams.

INTRODUCTION

Diagnostic imaging is an important tool to evaluate pancreatic neoplasms. Accurate detection and staging are essential for ensuring appropriate selection of patients who will benefit from surgery and for preventing unnecessary surgeries in patients with unresectable disease[1,2]. Ultrasound (US), multidetector computed tomography (MDCT) with multiplanar reconstruction and magnetic resonance imaging (MRI) can help to do a correct diagnosis[3,4].

A wide variety of anatomic variants and pathologic conditions exist that may mimic pancreatic neoplasms. Pancreas such as pancreas divisum or anular pancreas may cause enlargement of the pancreatic head and be mistaken for a tumoral mass. Non-distended adjacent bowel, gastric fundus, duodenal diverticula, duplications[2,5-7] accessory spleen or splenosis may also mimic a pancreatic mass[8]. Chronic pancreatitis may be indistinguishable from neoplasm on the basis of morphologic at MRI and MDCT[9] (Figure 1). Positron emission tomography (PET) with 2-[18F]-fluoro2-deoxy-d-glucose (FDG)/MRI fusion image significantly improved accu-

Table 1 Pancreatic tumors

Pancreatic tumors
Primary (95%)
Solid tumors
Pancreatic adenocarcinoma (85%-95%)
Pancreatic neuroendocrine tumor
Solid pseudopapillary tumor
Pancreatoblastoma
Pancreatic lymphoma
Cystic tumors
Serous cystadenoma
Mucinous cystic neoplasm
Intraductal papillary mucinous tumor
of the pancreas
Metastatic lesions (5%)

Figure 1 Multidetector computed tomography image. Multidetector computed tomography shows enlargement of the pancreatic head (arrow), with dilatation and beading of the pancreatic duct (arrowhead) and dilatation of the extra- and intrahepatic bile ducts. A focal calcification can also be visualized. These findings matched with the definite diagnosis of a chronic pancreatitis.

racy compared with that of PET/CT (in differentiating pancreatic cancer from benign lesions 96.6% *vs* 86.6%)[10].

Enlarged peripancreatic nodal chains and disease in surrounding structures can mimic pancreatic masses (gastric fundus neoplasm, small bowel tumors, renal or adrenal masses, *etc*.). The existence of fat planes between the nodes or tumoral masses and the pancreatic gland or displacement of the pancreas may be useful to distinguish these lesions from a pancreatic mass[6] (Figure 2). Choledochal cysts may simulate a cystic mass in the head of the pancreas[11].

True pancreatic masses can be classified in primary or metastatic lesions (Table 1).

PRIMARY PANCREATIC LESIONS

Primary pancreatic masses will be classified on the basis of its radiologic appearance in solid or cystic lesions.

SOLID LESIONS OF THE PANCREAS

Pancreatic adenocarcinoma

Pancreatic adenocarcinoma accounts for 85%-95% of all pancreatic malignancies and is the fourth leading cause of cancer-related deaths. Most patients are 60-80 years of age, and males are affected twice as often as females[3,4]. Of these tumors, 60%-70% are located in the pancreatic head, 10%-20% in the body, and 5%-10% in the tail. Diffuse glandular involvement occurs in 5% of cases[2,3]. Surgery is the only cure, with a postoperative 5-year survival rate of 20%[3,4]. Unresectable disease is seen at presentation in 75% of patients (Figure 3).

Dual-phase (arterial and portal) contrast material–enhanced MDCT is the established technique for evaluating pancreatic adenocarcinoma. Arterial phase imaging (performed 20-40 s after contrast agent injection) allows optimal visualization of the tumor and peripancreatic arteries (Figure 4). Portal phase imaging (performed 50-70 s after injection) is optimal for assessing the peripancreatic veins and detecting metastatic disease to the liver[3] (Figure 5). After intravenous contrast administration most tumors are hypoattenuating (Figure 6).

No pancreatic mass is visualized in 10% of cases, since the tumor may be isoattenuating. The presence and location of a mass may be inferred from secondary signs such as mass effect, an abnormal convex contour of the pancreas, ductal obstruction, and vascular invasion[2-4] (Figures 7 and 8). Tumors in the pancreatic head may cause dilatation of both common bile duct and the main pancreatic duct (MPD), known as the "double duct sign"; whereas tumors in the pancreatic body may cause upstream MPD dilatation (Figure 9A). A circumferential soft-tissue cuff around the peripancreatic vessels with loss of the perivascular fat plane denotes vascular invasion. A sensitivity of 84% and a specificity of 98% for invasion are reported if the tumor is contiguous with more than 50% of the vessel circumference[1] (Figure 9B). Other features suggesting vascular invasion include vessel deformity, thrombosis, and development of collateral vessels[12]. Cystic-necrotic degeneration, an uncommon feature of adenocarcinoma, is present in 8% of cases[13,14]. Metastases are most commonly found in the liver (Figure 5B) and peritoneum (Figure 9C)[2,3].

Adenocarcinoma has low signal intensity on T1 and T2 weighted MRI secondary to its scirrhous fibrotic nature (Figure 10). As at MDCT, the hypovascular tumor enhances less than the normal pancreas at MRI (Figure 11). MRI has better contrast resolution than MDCT and is superior in detecting small tumors and metastases[15]. Diffusion-weighted (DW) MRI allows the assessment of thermally induced random molecular motion in biologic tissues and generates representative apparent diffusion coefficient (ADC) values[16-18]. The use of DW MRI may allow earlier detection of pancreatic tumours, since these neoplasms have increased signal intensity on diffusion-weighted images and relatively low ADC values because of the restricted diffusion associated with fibrosis (Figure 12). In addition, DW MRI may be helpful in the detection of metastases in the liver and lymph nodes[16,17].

Endoscopic US has a recognized role in the detection and staging of small tumors. It can help detect masses as small as 0.2 cm. Endoscopic US can clarify equivocal find-
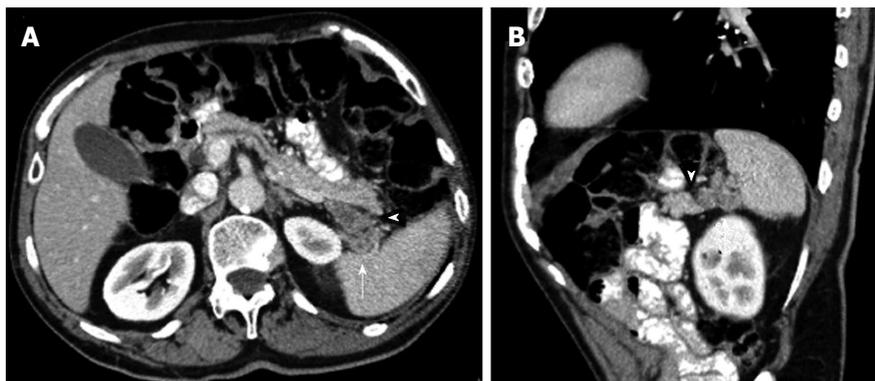
Figure 2 Axial contrast enhanced multidetector computed tomography image. A: Depicts a nodular peripancreatic mass localized between the pancreatic tail (arrowhead) and the splenic hilum (arrow), each well separated by fat planes; B: The sagittal reformatted contrast enhanced multidetector computed tomography image allows a better identification of the surrounding fat planes (arrow and arrowhead) enabling the exclusion of a pancreatic dependency. This mass actually turned out to be a tumoral implant of a gastric neoplasm.

Figure 3 Unresectability of a pancreatic adenocarcinoma. Contrast enhanced multidetector computed tomography (MDCT) image (A) and coronal reformation image (B) shows dilatation of the distal pancreatic duct caused by a hypodense tumor (arrow) in the pancreatic body. On plain film (C) and coronal reformation image on MDCT (D) of the same patient multiple lung metastases of his pancreatic carcinoma are evident - a definite criteria for unresectability.

ings at MDCT or MRI and allows biopsy of suspect lesions. Adenocarcinoma appears as an ill-defined, heterogeneous hypoechoic mass at endoscopic US[3] (Figure 13).

PET is an emerging technique for characterizing tissue on the basis of functional rather than morphologic information. The principle of FDG PET is that malignant tissues have greater uptake and retention of FDG than does normal tissue due to enhanced glucose metabolism. Pancreatic adenocarcinoma generally shows intense focal FDG uptake. The biggest potential impact of FDG PET is in the detection of small metastases, an area in which MDCT and MRI generally underestimate

lesions[3].

Pancreatic neuroendocrine tumor

Pancreatic neuroendocrine tumors (NETs) account for 1%-5% of all pancreatic tumors and typically manifest in patients aged 51-57 years. Most cases are sporadic, but association with syndromes such as multiple endocrine neoplasia type 1, von Hippel-Lindau syndrome, neurofibromatosis type 1, and tuberous sclerosis has been observed. Tumors tend to be multiple when associated with syndromes.

NETs are classified into functioning and nonfunc-
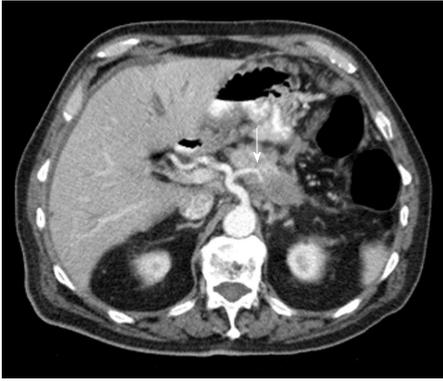
Figure 4 Axial contrast enhanced multidetector computed tomography image. Arterial phase imaging allows optimal visualization of the pancreatic neoplasm and peripancreatic arteries: the shown hypodense mass compromises the splenic artery (arrow). Pancreatic adenocarcinoma was proven by biopsy.


Figure 5 Contrast enhanced multidetector computed tomography image. A: In portal venous phase depicts a mass (arrow) in the pancreatic tail with permeability of the splenic vein (arrowhead); B: Focal round focal hypodensities with different sizes, localized in both hepatic lobules, represent metastatic spread to the liver. Pancreatic adenocarcinoma was proven by biopsy.

tioning tumors. Functioning tumors produce symptoms related to excessive hormone production. In general, functioning tumors manifest early in the course of disease. Nonfunctioning tumors manifest when they are large, due to mass effect. Risk of malignancy increases with tumor size (especially in tumors > 5 cm). Because of this fact 90% of nonfunctioning tumors are malignant at presentation[19].

Small tumors are generally solid and homogeneous, whereas larger tumors are heterogeneous and may show

Figure 6 Axial multidetector computed tomography image. Pancreatic tumor, localized in the pancreatic head (arrow), is hypodense in relation to the pancreatic parenchyma after contrast administration.

variable amounts of cystic-necrotic degeneration and calcification[3,19,20] (Figure 14).

NETs have a rich vascular supply and therefore enhance avidly during the arterial phase, enhancing more rapidly and intensely than the normal pancreas. That finding helps differentiate NETs from the more common adenocarcinoma which is hypovascular. Homogeneous enhancement is typical for small tumors (less than 2 cm), whereas larger lesions tend to show heterogeneous enhancement.

When NETs have a predominantly cystic component MDCT and MRI show a hypervascular enhancement in the nonnecrotic or nondegenerated portions of the tumor. Cystic areas are typically hyperintense at MRI on T2-weighted images (Figure 15).

Metastases to lymph nodes and solid organs such as the liver may have an enhancement pattern similar to that of the primary tumor (Figure 16). Cystic metastases to the liver may also be seen[3,19].

Solid pseudopapillary tumor

Solid pseudopapillary tumor (SPT) accounts for 1%-2% of all pancreatic tumors. It is most common in young females (mean age, 25 years)[21]. SPT has a low malignant potential with an excellent prognosis following complete resection.

SPT is typically a large (mean, 9 cm), slow-growing, well-encapsulated mass[21,22]. It most commonly occurs in the pancreatic tail. SPT has a tendency to displace rather than invade surrounding structures and rarely causes obstruction of the bile duct or pancreatic duct. MDCT usually demonstrates a well-encapsulated lesion with varying solid and cystic components owing to hemorrhagic degeneration[23]. Hemorrhage may progress to cystic changes within the lesions in approximately 20% of cases. Degenerated areas may mimic certain features of larger NETs. However, the peripheral portions of solid and papillary epithelial neoplasms do not demonstrate the hypervascularity typical of NETs[21]. SPT shows peripheral heterogeneous enhancement with central cystic spaces[24,25].

MRI typically demonstrates a well-defined lesion with
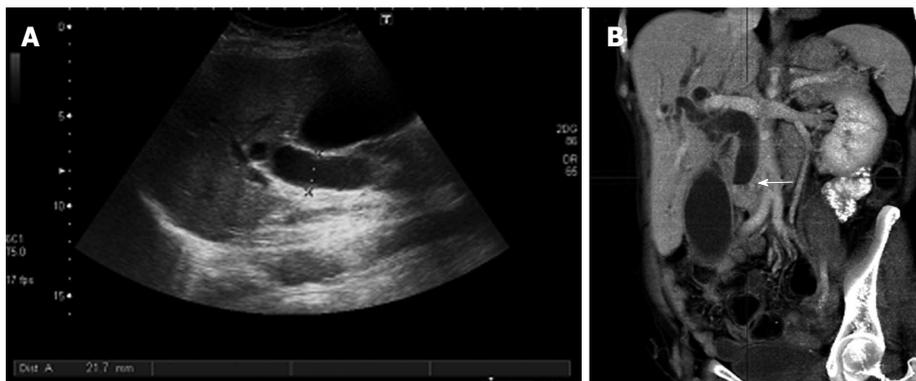
Figure 7 Indirect signs of pancreatic neoplasms. Transverse ultrasound image (A) shows a markedly dilated common bile duct, also seen on the coronal reformation image of multidetector computed tomography (B) where the dilated duct terminates abruptly at the level of the pancreatic head (arrow).

Figure 8 Endoscopic retrograde cholangiopancreatography. A short segment of narrowing causing stenosis of the common bile duct was recognized (arrow), without affection of the main pancreatic duct (arrowhead). Pancreatic adenocarcinoma was proven by biopsy.

heterogeneous signal intensity on T1- and T2-weighted images. Peripheral calcification is present in 30% of cases[21]. The pseudocapsule (composed of compressed pancreatic tissue and reactive fibrosis) has low attenuation at MDCT and low signal intensity at T1- and T2-weighted MRI.

Internal hemorrhagic and cystic degeneration is the hallmark of SPT due to the fragile vascular network of the tumor[3,26]. Although most SPTs exhibit benign behavior, malignant degeneration does occur. Metastases are uncommon, occurring in 7%-9% of cases, mostly to the liver, omentum, and peritoneum[27].

Pancreatoblastoma

Pancreatoblastoma accounts for 0.2% of all pancreatic tumors and is the most common pancreatic tumor in young children (mean 5 years)[3,28]. Pancreatoblastoma rarely occurs in adults; when it does, however, the tumor is generally more aggressive. The serum alpha-fetoprotein level is elevated in 25%-33% of cases[29].

Pancreatoblastoma is typically slow growing and generally manifests as an asymptomatic large mass (mean, 10 cm). Because of the large size of the mass at presentation, in 50% of cases it is not possible to identify the
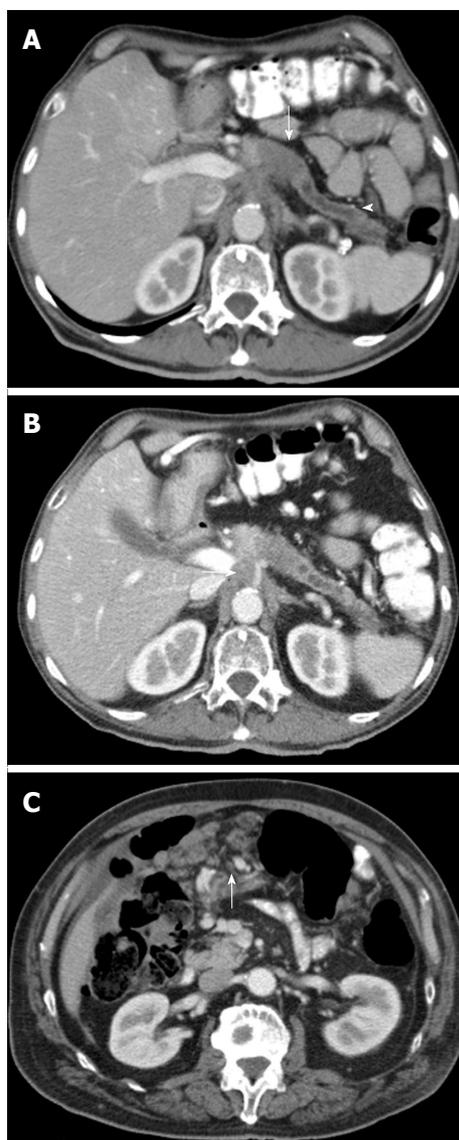
Figure 9 Axial contrast enhanced multidetector computed tomography image A: Focal hypodense mass in the body of the pancreas (arrow), with upstream dilatation of the main pancreatic duct (arrowhead). Pacreatic adenocarcinoma was histologically proven; B: Image depicts a circumferential soft tissue cuff around the celiac trunk according to vascular invasion (arrow); C: Image shows multiple peritoneal metastases in a patient with a pancreatic tumor (arrow).
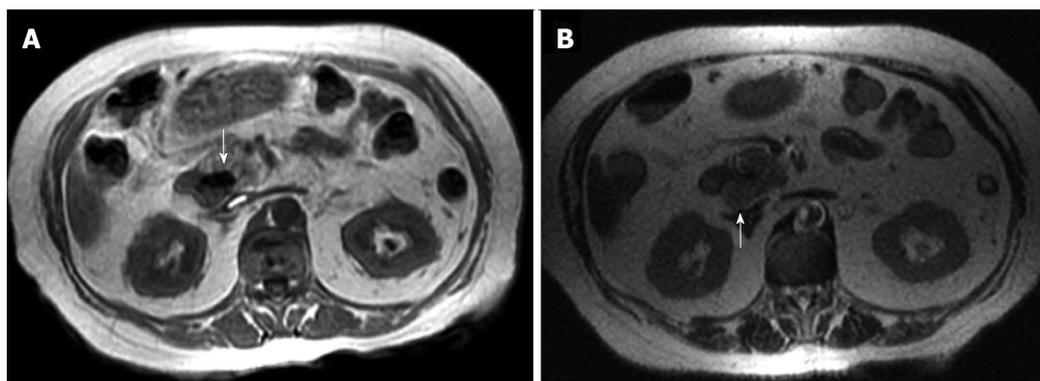
Figure 10 Adenocarcinoma has low signal intensity on T1 (A) and T2 (B) weighted magnetic resonance imaging (arrows).
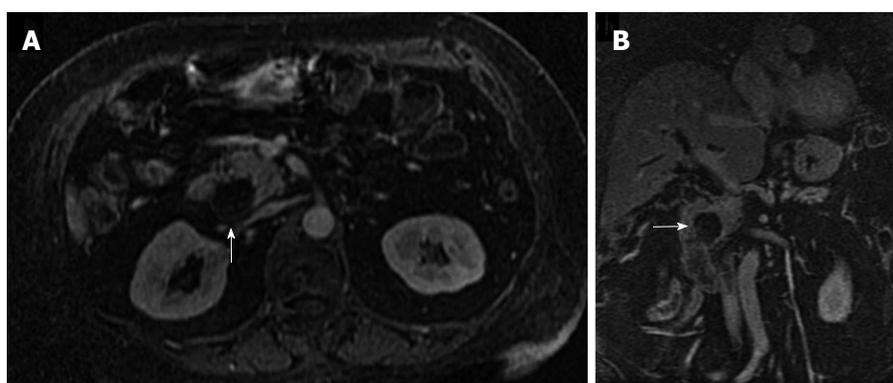
Figure 11 Axial arterial-phase gadolinium-enhanced T1-weighted fat-suppressed gradient-recalled echo magnetic resonance imaging (A) and coronal re-formatted (B) show no enhancement of the hypovascular tumor in the pancreatic head (arrow). Pancreatic adenocarcinoma was proven by biopsy.
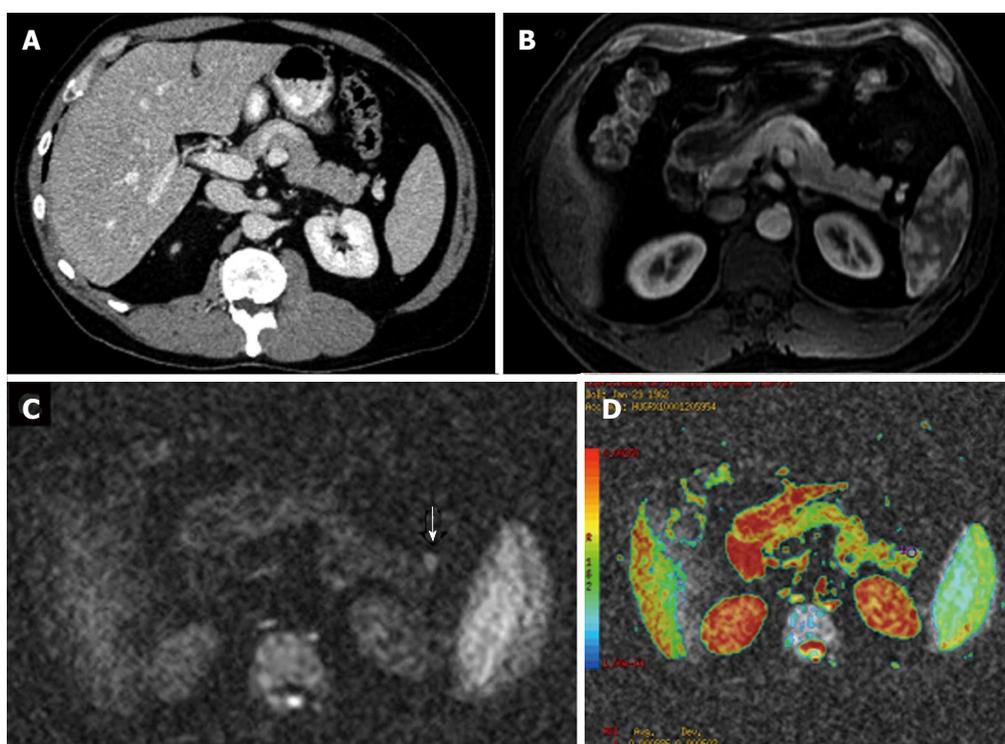
Figure 12 Use of diffusion-weighted magnetic resonance imaging in the earlier detection of pancreatic tumours. Axial contrast enhanced multidetector computed tomography image (A) and axial arterial-phase gadolinium-enhanced T1-weighted fat-suppressed gradient-recalled echo magnetic resonance image (B) do not depict any abnormality. Axial diffusion-weighted magnetic resonance imaging (C) demonstrates a focal increased signal intensity (arrow) and low apparent diffusion coefficient values in the color coded images (D).
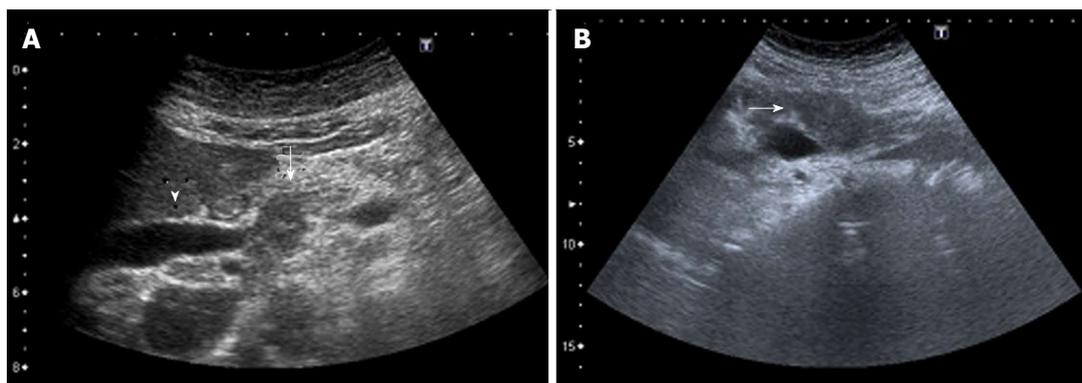
Figure 13 Ultrasound images (A, B) of an ill-defined, heterogeneous hypoechoic mass (arrow) in the pancreas obstructing the common bile duct (arrowhead).
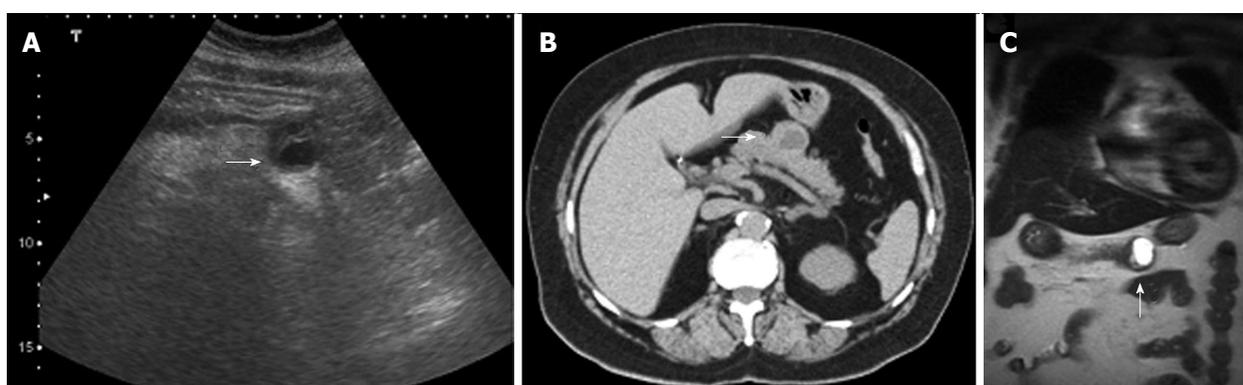
Figure 14 Pancreatic neuroendocrine tumor. Ultrasound images (A), axial unenhanced multidetector computed tomography and coronal magnetic resonance T2-weighted image show a round, heterogeneous mass, localized in the pancreatic body, with variable amounts of cystic-necrotic degeneration (arrows).
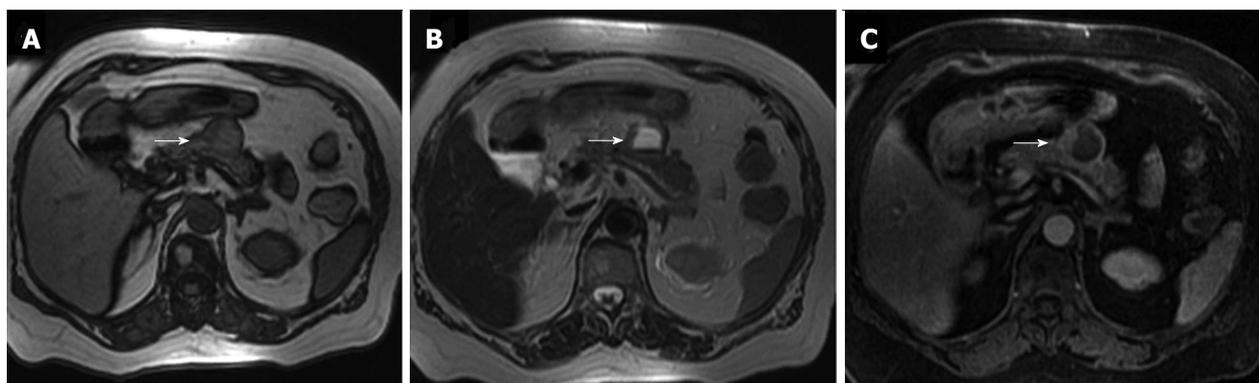
Figure 15 Same patient shown in figure 14. Magnetic resonance axial gradient T1 out-of-phase image (A) and T1 fat-suppressed sequence (C) show a hypointense signal in the liquid component of the lesion whereas it reveals a hyperintense signal in the T2-weighted sequence (B) (arrows).

organ of origin at radiology[30]. Therefore, differentiation from other pediatric tumors arising from adjacent organs (*e.g.*, neuroblastoma, Wilms tumor, hepatoblastoma) is challenging, and biopsy is generally required to establish the diagnosis. Metastases occur mostly to the liver.

At US, the mass is heterogeneous with hypoechoic cystic spaces and hyperechoic internal septa[28]. At MDCT, pancreatoblastoma generally manifests as a multiloculated inhomogeneous mass with enhancing septa[28]. On MRI the tumor has low to intermediate signal intensity on

T_1- and high signal intensity on T_2-weighted images, and shows mild contrast enhancement.

Pancreatic lymphoma

Pancreatic lymphoma is most commonly a B-cell subtype of non-Hodgkin lymphoma. Secondary lymphoma is the dominant form and is the result of direct extension from peripancreatic lymphadenopathy. Primary pancreatic lymphoma is rare, representing 0.5% of pancreatic tumors. It is more common in immunocompromised patients[31].
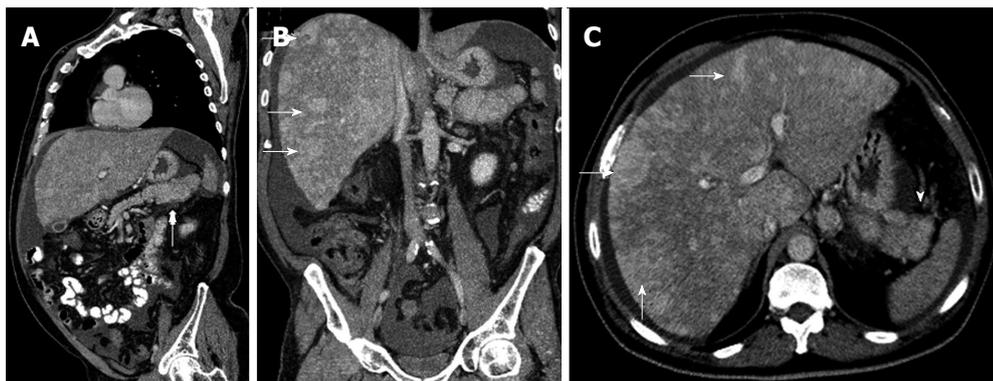
Figure 16 Sagittal multidetector computed tomography image. A: A heterogeneous pancreatic mass (arrow); B, C: Coronal (B) and axial (C) multidetector computed tomography images show multiple hypervascular metastases in the liver (arrows), showing the same enhancement pattern of the primary mass. Neuroendocrine pancreatic tumor and metastases were histologically proven.

Two morphologic patterns of pancreatic lymphoma are recognized: a focal well-circumscribed form and a diffuse form. The focal form occurs in the pancreatic head in 80% of cases and has a mean size of 8 cm. It typically has uniform low attenuation at MDCT. At MRI, it has low signal intensity on T_1- and intermediate signal intensity on T_2- weighted images and shows faint contrast enhancement. The diffuse form is infiltrative leading to glandular enlargement and poor definition, features that can simulate the appearance of acute pancreatitis[32,33].

CYSTIC LESIONS OF THE PANCREAS

Cystic lesions accounts for 10%-15% of all pancreatic neoplasms and represents < 5% of all malignant pancreatic tumors.

Unilocular cysts are well defined lesions without internal septa, calcification or internal soft-tissues nodules. When small (< 3 cm), these lesions are almost always benign. It is suggested to do serial imaging at 6-mo intervals for the first year and annual follow-up for a period of three years. If the cyst remains stable and the patient asymptomatic no further workup in needed[34].

Pseudocyst (encapsulated fluid collections without necrosis after 4 wk from onset of acute pancreatitis) is the most common unilocular cyst[34,35]. It is important to ask for the patient's history because a cystic lesion in a patient with a clinical history of pancreatitis is almost always a pseudocyst.

Imaging studies shows a rounded cystic mass with a thick wall. After intravenous contrast administration mild wall enhancement is demonstrated (Figure 17). If we detect a solid intracystic component, the lesion is not a pseudocyst. Other image findings that support this diagnosis are inflammation, atrophy or pancreatic calcifications. Cystic neoplasm may appear as uni or multilocular masses.

Serous cystadenoma
It is a benign lesion which typically occurs in older women. The cystic components range from millimeters to 2 cm. When the lesion grows a central scar and coarse calcification may be seen (30%). This calcified scar is highly specific and virtually pathognomonic[36] and is best demostrated at CT.

MRI shows a cluster of small cyst without visible communication within the cyst or the pancreatic duct. These cysts are hyperintense on T_2-weighted images. Central calcified scar is seen as a signal void at MRI (Figure 18). Enhancement of fibrous septa between the cysts are seen on delayed images.

Mucinous cystic neoplasm (mucinous cystadenoma/ cystadenocarcinoma)
This lesion has female predominance (80%) in their sixth decade of life[37]. Mucinous cystadenoma preferentially involves the body and pancreatic tail and do not communicate with the pancreatic duct.

Cross-sectional imaging is ineffective for differentiating between mucinous cystic neoplasms with and without malignant epithelium, except in cases with invasion of adjacent organs, vascular invasion, or metastatic disease. The presence of intracystic enhancing soft tissues are suspicious for malignancy. Peripheral eggshell calcifications are not frequent (16%) but such finding is specific and has a highly predictive value for malignancy.

On US mucinous cystic neoplasms appear as hypoechogenic multilocular or, less commonly, unilocular masses with posterior acoustic enhancement. Internal septations are usually visualized and better demonstrated at US than at CT[36-40].

CT shows a round to slightly lobulated mass that is well encapsulated with smooth external margins. Because the cyst contents can vary in attenuation according to the degree of hemorrhage or protein in the mucoid cysts, different levels of attenuation may be seen within the cyst cavities[37,39,41-44] (Figure 19). After intravenous contrast administration septa and peripheral wall enhancement are detected.

At MR the lesion is hypointense on T_1- and hyperintense on T_2-weighted images. This lesion may be hyperintense on T_1-weighted images due to mucinous content.
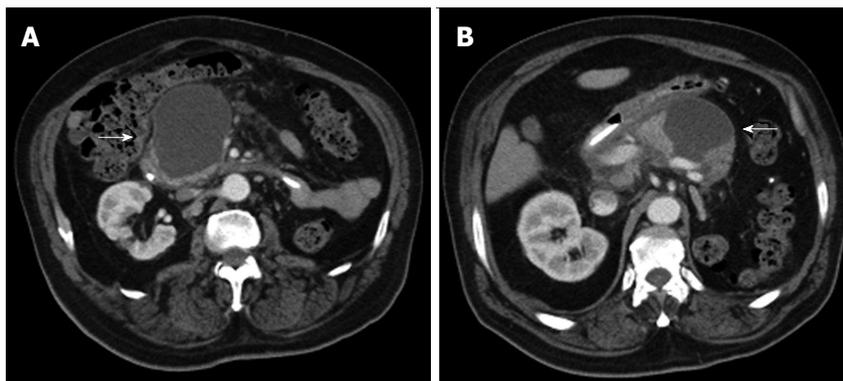
Figure 17 Axial contrast enhanced multidetector computed tomography images (A, B) reveal a homogeneously hypodense intraparenchymal fluid collection of the pancreas without any non-liquefied material in it, encapsulated completely by a thin slightly hyperdense layer (arrows). These findings are compatible with a pseudocyst in a patient with a clinical history of pancreatitis.
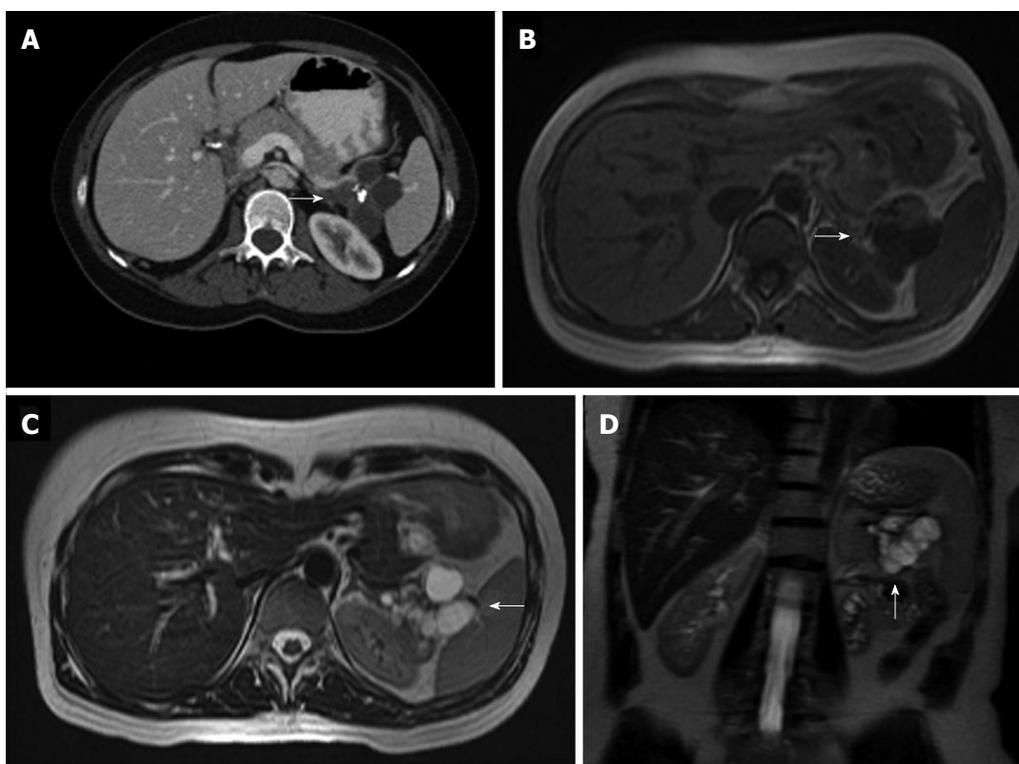
Figure 18 Axial nonenhanced multidetector computed tomography image. A: A polylobulated cystic lesion with a coarse calcification in its center (arrow), which is the phathognomonic central scar for serous cystadenoma; B-D: Magnetic resonance imaging show a cluster of small cysts (arrows), which are hypointense in T1-weighted images (B) and hyperintense in T2-weighted images (C, D), without visible communication within the cyst or the pancreatic duct. A central signal void is also identifiable.

Intraductal papillary mucinous tumor of the pancreas

Intraductal papillary mucinous tumor of the pancreas (IPMN) are most frequent indentified in elderly men. The most important features are the presence of mucin-producing tumor and cystic dilation of the main pancreatic duct, its branches or both[45,46]. The dilated ducts often contain profuse mucin. In the past, many IPMTs may have been misdiagnosed as chronic pancreatitis because of their generally benign behavior.

IPMNs may be classified as benign or malignant on the basis of the degree of dysplasia[47-50].

Preoperative determination of the presence or absence of associated invasive carcinoma is crucial; when invasive carcinoma is present, the surgical procedure may be modified to include resection of regional lymph nodes.

Main duct IPMNs are more likely to be malignant. IPMNs are frequently multifocal, and 5%-10% involve the entire pancreas.

When CT reveals a pancreatic solid mass in patients with IPMN, the lesion is probably invasive carcinoma. Other imaging features suggestive of invasive carcinoma in IPMN are the large size of the mass (> 3.5 cm), presence of mural nodules, dilatation of the main pancreatic
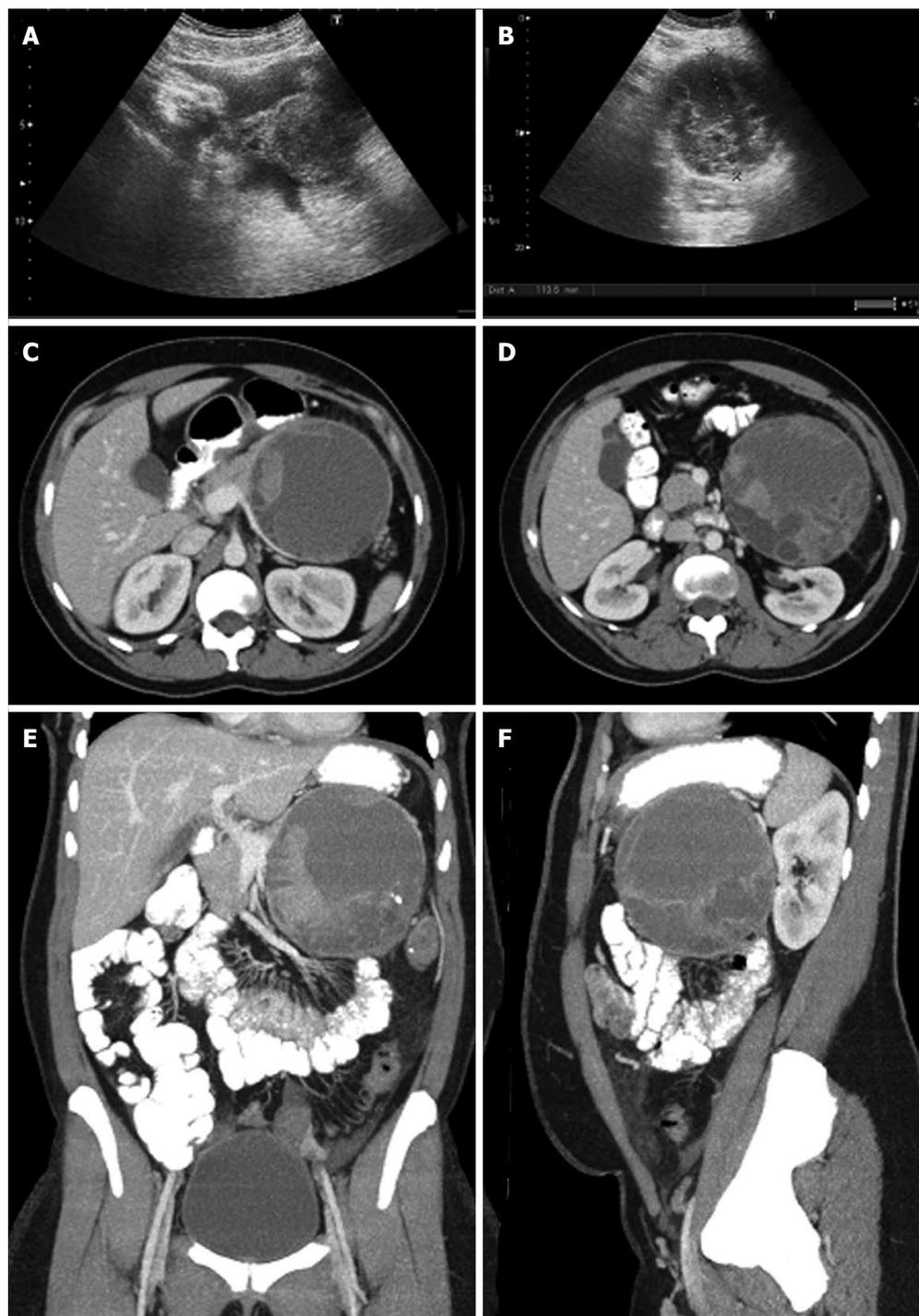
Figure 19 Ultrasound and multidetector computed tomography images. On ultrasound (A, B) a hypoechogenic multilocular mass with well-definable internal spetations and posterior acoustic enhancement can be seen. Contrast-enhanced multidetector computed tomography images (C-F) show a big round to slightly lobulated mass with an enhancing capsule and different levels of attenuation within the cyst cavities are seen. Some enhancing components are also detectable.

duct > 15 mm and multifocal involvement[49,51].

MRI is better than CT for evaluating ductal communication[52,53]. Dilatation of main pancreatic duct or multiple side branches on T_2-weighted images is the most common imaging finding[54]. Demonstrating ductal communication can be useful to differentiate between IPMNs and mucinous cystadenoma (the latter has no communication with the pancreatic ductal system) (Figure 20).

Three-dimensional contrast-enhanced ultrasonog-

raphy showed similar results as compared with MRI in evaluating "IPMNs" smaller than 1 cm of diameter or greater than 2 cm[55].

METASTASES TO THE PANCREAS

Pancreatic metastases account for 2%-5% of all malignant neoplasms. Metastases are most frequently from renal cell carcinoma and lung carcinoma[56]. The progno-
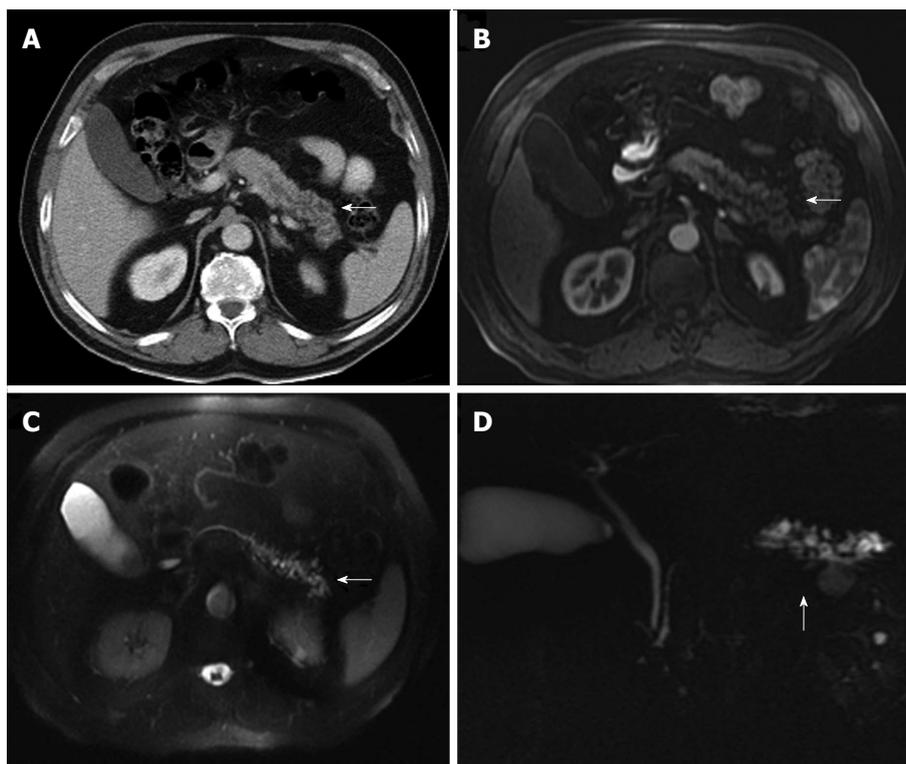
Figure 20 Multidetector computed tomography image. A: Cystic dilatation of the main pancreatic duct and some of its branches in the pancreatic tail. Ductal communication with the tumor cannot be clearly identified; B-D: In contrast-enhanced axial T1 (B) and T2-weighted (C) magnetic resonance images and in magnetic resonance imaging cholangiography (D) ductal communication can be easily detectable.

sis is generally more favorable than that for pancreatic adenocarcinoma[3] (Figure 21).

Three morphologic patterns of involvement are recognized: solitary (50%-70%), multifocal and diffuse[56,57]. At contrast-enhanced CT and MR imaging, the appearances of pancreatic metastases closely resemble that of primary carcinoma but pancreatic adenocarcinoma generally manifests as a hypoenhancing mass, whereas metastases show either peripheral enhancement (in lesions > 1.5 cm) or, less commonly, homogeneous enhancement (smaller lesions)[56,58,59].

Cystic metastases to the pancreas cannot be differentiated from mucinous cystic neoplasms radiographically. Ovarian carcinoma metastases are the most likely to manifest as a predominantly cystic mass.

A known history of primary malignant disease, combined with the presence of other metastatic foci, are helpful clues in making the diagnosis.

INTRAOPERATIVE ULTRA-SONOGRAPHY OF THE PANCREAS

Up to 40% of patients with pancreatic adenocarcinoma judged resectable at CT are found to have unresectable lesions at surgery[60,61]. Laparoscopy intraoperative US may be useful before open surgical resection to decrease the number of patients who undergo needless open surgery for resection of a tumor that ultimately proves unresectable[62]. Pancreatic adenocarcinoma appears at intraoperative US as a hypoechoic mass with ill-defined

margins[60].

EVALUATION OF THE POSTOPERATIVE PANCREAS

The most common complications of the Whipple procedure are delayed gastric emptying, pancreatic fistulas, wound infection, abdominal abscess, intraabdominal bleeding, and anastomotic leakage. A pancreaticojejunal fistula is diagnosed clinically on the basis of the detection of amylase-rich fluid in the drainage. Anastomotic leaks usually occur at the pancreaticojejunal anastomosis during first 2 wk after pancreatoduodenectomy and these leaks can be diagnosed on the basis of the presence of oral contrast material in the peritoneal cavity and are associated with peripancreatic fluid collections[63,64].

Locally recurrent disease is sometimes difficult to depict on the earliest postoperative images. Locally recurrent disease appears as an infiltrating mass with soft-tissue attenuation, perineural invasion and encasement of the mesenteric vessels[65]. Perivascular cuffing in the mesenteric fat is likely inflammatory in patients with negative surgical margins and should not be mistaken for local recurrence[63].

CONCLUSION

The knowledge of some of the most important characteristic key findings of pancreatic tumors may facilitate radiologists, and especially radiographers in training,

Figure 21 Oblique reformatted enhanced multidetector computed tomography image reveals a well-defined round mass in the pancreas, slightly hypodense to the pancreatic parenchyma. Pancreatic metastases from melanoma was proven. Note the liver concomitant metastases.

to do an accurate detection and staging of pancreatic neoplams in order to ensure an appropriate selection of patients who will benefit from surgery and prevent unnecessary surgeries in patients with unresectable disease.

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Jose Manuel Ramia, MD, PhD, FACS, *Series Editor*

Role of endoscopic ultrasound in the diagnosis of pancreatic cancer

Juana Gonzalo-Marin, Juan Jose Vila, Manuel Perez-Miranda

Juana Gonzalo-Marin, Unit of Endoscopy, Department of Gastroenterology, Quirón Hospital, 29603 Marbella, Spain

Juan Jose Vila, Unit of Endoscopy, Department of Gastroenterology, Complejo Hospitalario de Navarra, 31008 Pamplona, Spain

Manuel Perez-Miranda, Department of Gastroenterology, Hospital Universitario Rio Hortega, 47012 Valladolid, Spain

Author contributions: Vila JJ and Perez-Miranda M performed the research for the most relevant manuscripts for the review and were also involved in editing the final manuscript; Gonzalo-Marin J performed the review and wrote the manuscript.

Correspondence to: Manuel Perez-Miranda, MD, Department of Gastroenterology, Hospital Universitario Rio Hortega, Dulzaina street, 47012 Valladolid, Spain. mperezmiranda@saludcastillayleon.es

Telephone: +34-98-3420400 Fax: +34-98-3215365

Abstract

Endoscopic ultrasonography (EUS) with or without fine needle aspiration has become the main technique for evaluating pancreatobiliary disorders and has proved to have a higher diagnostic yield than positron emission tomography, computed tomography (CT) and transabdominal ultrasound for recognising early pancreatic tumors. As a diagnostic modality for pancreatic cancer, EUS has proved rates higher than 90%, especially for lesions less than 2-3 cm in size in which it reaches a sensitivity rate of 99% *vs* 55% for CT. Besides, EUS has a very high negative predictive value and thus EUS can reliably exclude pancreatic cancer. The complication rate of EUS is as low as 1.1%-3.0%. New technical developments such as elastography and the use of contrast agents have recently been applied to EUS, improving its diagnostic capability. EUS has been found to be superior to the recent multidetector CT for T staging with less risk of overstaying in comparison to both CT and magnetic resonance imaging, so that patients are not being ruled out of a potentially beneficial resection. The accuracy for N staging with EUS is 64%-82%. In unresectable cancers, EUS also plays a therapeutic role by means of treating oncological pain through celiac plexus block, biliary drainage in obstructive jaundice in patients where endoscopic retrograde cholangiopancreatography is not affordable and aiding radiotherapy and chemotherapy.

Core tip: In this article, the role of endoscopic ultrasonography as a diagnostic, staging and therapeutic procedure in patients with pancreatic cancer is discussed and all the current knowledge on this subject is summarized, providing the reader with a quick update.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer-related death in men and the first leading cause in women, with an approximate incidence of ten per 100000 population per year[1,2].

Multiple-imaging modalities are used in combination in the diagnosis and staging of pancreatic cancer: transabdominal ultrasound, computed tomography, magnetic resonance and endoscopic retrograde cholangiopancreatography (ERCP).

The prognosis of pancreatic cancer is dismal, with a 1 and 5 year survival rate at all stages at diagnosis of 24% and 5%, respectively, according to the latest from the American Cancer Society[3]. Without treatment, the average survival of patients with pancreatic cancer is four months[4]. Endoscopic ultrasound (EUS) could be a good imaging technique for a better selection of patients for an effective curative treatment.

In addition, by the time pancreatic cancer manifests symptoms that demand medical attention, it has already spread to the point of unresectability in nearly 80%-90% of patients because of metastatic disease[4,5]. It is especially in these patients where the therapeutic spectrum of EUS is growing. Treatment of oncological pain through celiac plexus block, biliary drainage in obstructive jaundice in patients where ERCP is not affordable and aiding radio-therapy and chemotherapy are some examples of this.

Therefore, EUS has several roles in the widespread sphere of pancreatic cancer. The introduction of EUS in the 1980s was received with great enthusiasm because of the improved information it could provide on the pancreas by overcoming the limitations associated with the use of transabdominal ultrasound. EUS with or without fine needle aspiration (FNA) has been shown to be a cost-effective technique for evaluating pancreatobiliary disorders, particularly where others have failed[6], and has a higher diagnostic yield than positron emission tomography (PET), computed tomography (CT) and trans-abdominal ultrasound for recognizing early pancreatic tumors[1,2].

Pancreatic cancer diagnosis can be made with accurate sensitivity and specificity by EUS because of its inherent advantage of a high-frequency transducer placed in close proximity to the tumor which provides a high resolution image, especially with the incorporation of contrast enhanced images in the last years, making possible a differential diagnosis with other pathologies, such as chronic pancreatitis and neuroendocrine tumors[7], and a histological confirmation using EUS-FNA

THE ROLE OF EUS FOR DIAGNOSIS OF PANCREATIC CANCER

Numerous studies indicate that EUS is highly sensitive for the detection of pancreatic tumors with rates higher than 90%[8], especially for lesions less than 2-3 cm in size in which it reaches a sensitivity rate of 99% *vs* 55% for CT[9,10]. Although the sensitivity for tumor detection is high, it is also important to note that it has a very high negative predictive value (NPV)[11,12]. This is quite important for clinicians because it means that EUS could reliably exclude pancreatic cancer. On the other hand, this evidence comes from one study only and certain conditions explained further on in the text may hinder a diagnosis of pancreatic cancer.

EUS also has the ability to provide FNA which has made it essential in the evaluation of patients with solid pancreatic lesions since most patients require a tissue di-

agnosis before treatment.

Certain tumor extrinsic conditions exist that may hinder the identification of pancreatic cancer[13]: chronic pancreatitis with a severe inhomogeneous echotexture, diffuse infiltration by tumor, prominent ventral/dorsal division and acute pancreatitis lasting less than 4 wk.

THE ROLE OF EUS IN THE DIFFERENTIAL DIAGNOSIS OF PANCREATIC CANCER

Differential diagnosis of solid pancreatic masses remains a challenge. Dynamic contrast-enhanced CT is the most widespread imaging technique for this purpose and has been considered the most comprehensive tool for diagnosis and surgical staging of pancreatic malignancies[5]. Despite all the advances with the multidetector helical CT scan, differential diagnosis between mass-forming chronic pancreatitis, ductal adenocarcinoma and autoimmune pancreatitis based on only CT image is still difficult[14,15].

Magnetic resonance imaging (MRI) could also be useful in the differentiation of pancreatic solid masses but several studies have demonstrated that is less sensitive than CT and EUS[16,17]. The administration of secretin during magnetic resonance cholangio-pancreatography can be useful, enhancing the image of the main pancreatic duct, providing pancreas function and duct shape information as dilation[18].

Currently, ERCP has no clinical role in the diagnosis and staging of pancreatic cancer. Indirect findings such as combined dilation of the bile and the pancreatic duct or abrupt cutoff in the main pancreatic duct or a solitary long stricture of the pancreatic duct could raise suspicion of malignant disease but may also be observed in chronic pancreatitis.

PET is an image modality which relies upon detection of functional activity rather than lesion size alone. Tumors have enhanced glucose uptake and normal pancreas has low glucose utilization rate, fluorodeoxyglucose labelled with radioactive fluorine ([18]FDG-PET) readily accumulates in malignant cells and can be detected by a PET camera[19]. However, the role of [18]FDG-PET in evaluation of primary pancreatic adenocarcinoma has not been established in evaluating tumor response to neoadjuvant chemoradiotherapy or in the evaluation of recurrent disease after surgical resection.

EUS is considered to be one of the most accurate methods for diagnosis of inflammatory, cystic and neoplastic diseases of the pancreas[4,20,21] and recent studies recommend it for the differential diagnosis of solid pancreatic masses, although accuracy in differentiation between benign inflammatory masses and malignant tumors of pancreas has not been higher than 75%[22-27].

In a study by Eloubeidi *et al*[28], 101 patients with solid pancreatic masses underwent an average of 4 needle passes with EUS-FNA, resulting in a sensitivity of 95%, specificity of 95%, positive predictive value (PPV) of 100% and NPV of 85.2%.

EUS-FNA can be made using different types of

needles. Small calibre needles (25 G) have a similar cytology yield as large calibre needles (19 G) with less blood contamination and the advantage of greater flexibility for difficult-to-reach areas such as the uncinate process[29]. The prospective study by Sakamoto *et al*[30] showed that 25-gauge was the best choice of needle for cytological diagnosis of solid pancreatic lesions and, in cases in which a histological diagnosis is desired, the 22-gauge FNA needle and 19-gauge trucut needle may be an advantage in head/uncinate and body/tail lesions, respectively.

On-site cytopathology for some investigators is deemed a superior standard of care with the provision of opportunity for real-time interpretation of samples[31,32] so that it improves the diagnostic yield of EUS-FNA independent of the number of needle passes undertaken for tissue sampling[33]. If this cannot be provided, 5-6 passes for pancreatic masses and 2-3 passes for peripancreatic lymph nodes and metastases will provide the maximum yield[34]. Also, having an experienced cytopathology technician or to specifically train a EUS nurse to prepare and determine cellular adequacy for each sample[33] is helpful in these cases. In cases in which initial cytology is indeterminate or non-diagnostic, the literature supports reattempting EUS-FNA and combining routine cytology with fluorescence in situ hybridization (FISH) and K-ras/p53 analysis to improve the diagnostic yield. This combination yields 87.9% sensitivity, 93.8% specificity, 96.7% PPV, 78.9% NPV and 89.8% accuracy in the Reicher and colleagues retrospective study[35]. FISH plus K-ras analysis correctly identified 60% of atypical FNAs with a final malignant diagnosis.

EUS is considered a safe procedure with complication rates as low as 1.1%-3%[36]. Commonly reported complications include bleeding (1%-4%), pancreatitis (1%-2%), perforation (0.03%)[37] and rarely tumor seeding after EUS-guided FNA[38-42]. The risk of tumor seeding along the needle tract has been a concern especially in Japan. Although the reported incidence of tumor seeding after EUS-FNA is scarce, the indication of EUS-FNA for small lesions located at pancreas body/tail where the aspiration route will not be included in the resection area needs to be carefully considered. When pancreatic head lesions are evaluated by FNA, there is a theoretical risk of cancer seeding, but this has never been reported after EUS-FNA because after a Whipple procedure, the potential sites of seeding are removed. As for patients with unresectable disease, most die of disease progression before any seeding is detected. If the decision is to proceed to EUS-FNA, patients must be fully aware of the remote risk of seeding to the gastric wall[39]. There are two cases of tumor seeding along a EUS-FNA tract in pancreatic adenocarcinoma and both were pancreatic tail adenocarcinomas[39,40]. The only other two reports related to tumor seeding after EUS-FNA were peritoneal dissemination after EUS-FNA of pancreatic intraductal papillary mucinous neoplasia[41] and metastatic melanoma[42]. Whether this risk is increased by the needle size or number of passes remains uncertain.

The sensitivity of EUS-FNA for malignancy in patients with chronic pancreatitis is lower compared to when the surrounding parenchyma is normal[27,43-47]. Studies by Fristcher-Ravens *et al*[27] and Varadarajulu *et al*[44] found a sensitivity of 54% and 73.4% in parenchymas with chronic inflammation *vs* 89% and 91.3% in normal parenchyma respectively ($P = 0.02$). A systematic review of 53 studies estimated a NPV of EUS-FNA in the diagnosis of pancreatic adenocarcinoma as 60%-70%[48] which makes a new function mandatory in cases where the first EUS-FNA has been benign. The Procore® histology needle has been designed in order to optimize tissue sampling of EUS-FNA, allowing a histological evaluation with an overall accuracy of 89.4% in solid pancreatic lesions[49].

Recently, quantitative EUS elastography (QE-EUS) has been developed in an attempt to make the elastography interpretation less subjective than the old qualitative EUS-elastography. In the Iglesias-Garcia *et al*[23] study with 86 patients with solid pancreatic masses, the strain ratio (ratio of elasticity in the target area over soft referent tissue) was significantly higher among patients with malignant pancreatic tumors compared to those with inflammatory masses. Normal tissue showed a mean strain ratio of 1.68 (95%CI: 1.59-1.78), inflammatory masses 3.28 (95%CI: 2.61-3.96) and pancreatic adenocarcinoma 18.12 (95%CI: 16.03-20.21) ($P < 0.001$). The sensitivity and specificity of the strain ratio for detecting pancreatic malignancies in solid masses using a cut off value of 6.04 were 100% and 92.9% respectively, higher rates than obtained with qualitative elastography (100% and 85.5% respectively)[50].

Contrast-enhanced EUS (CEH-EUS) is performed with the application of contrast agents. Numerous US contrast agents (UCAs) are commercially available. Levovist®, the first agent for general use, is made of a galactose microcrystal filled with air bubbles which, shattering under a high sound pressure, emits pseudo-Doppler signals. With the development of second UCAs (Sonovue® and Sonazoid®) which contain inert gases with low solubility in water, the stability and duration of the contrast and real-time vascular images have been increased. The risk for drug allergy is small because of the small molecular weight of microbubbles and they are also applicable for patients with liver and renal dysfunctions because it is excreted by exhalation[51,52]. Most carcinomas, neuroendocrine tumors and inflammatory pseudotumors are simply depicted as hypoechoic masses, but the use of contrast agents in EUS has been shown to improve the characterization of the vasculature inside the organ of interest, to better delineate such hypoechoic masses. According to published reports, hypoenhancing masses were regarded as a sign of malignancy in CEH-EUS. The first feasibility study reported good values of sensitivity, specificity and accuracy for the differential diagnosis between adenocarcinoma and focal chronic pancreatitis[53]. This was further confirmed in two other studies by Sakamoto *et al*[54] and Dietrich *et al*[55] in which adenocarcinomas showed hypoenhancement compared with neuroendocrine tumors and pseudotumoral (mass-forming) pancreatitis, which

showed isoenhancement or hyperenhancement. Fukusawa *et al*[56] reported a prospective study, concluding that in most cases of pancreatic adenocarcinoma, CEH EUS exhibits a hypoperfusion pattern compared with the adjacent normal pancreatic tissue, whereas autoimmune pancreatitis/chronic pancreatitis exhibits iso-perfusion and pancreatic neuroendocrine tumors (PNET) exhibit a hyperperfusion pattern[56]. Fusaroli *et al*[57] found that a hypoenhancing mass with a inhomogeneous pattern diagnosed pancreatic adenocarcinoma with a sensitivity of 96% and more accuracy than standard EUS. Hyper-enhancement specifically excluded adenocarcinoma (98%), although with a low sensitivity. Seicean *et al*[58] introduced the use of quantitative CEH-EUS for differential diagnosis between pancreatic cancer and chronic pancreatitis, with the index of contrast uptake lower in adenocarcinoma compared to cases with mass-forming chronic pancreatitis. Also, using pulsed Doppler could help with the differential diagnosis between adenocarcinomas and chronic pseudotumoral pancreatitis. Pancreatic adenocarcinomas show mainly arterial-type signals and chronic pseudotumoral masses show both arterial-type and venous-type signals[59]. The first meta-analysis that summarized the available evidence of the diagnostic performance of CEH-EUS for the differential diagnosis of pancreatic adenocarcinomas showed that CEH-EUS had a pooled sensitivity of 94% (95%CI: 91-95) and a pooled specificity of 89% (95%CI: 85-92), so finding a hypoenhancing lesion was a sensitive and accurate predictor of pancreatic adenocarcinoma[60]. The variation in this study in comparison with Fusaroli *et al*[57] may have occurred because more patients with severe chronic pancreatitis were enrolled in the Fusaroli *et al*[57] study, which may have altered the enhanced pattern of pancreatic adenocarcinomas. Severe forms of chronic pancreatitis mean less intense intralesional "parenchymographic" enhancement and fibrosis resulting in decreasing vascular flow[62-64]. Iglesias-Garcia *et al*[65] compared the aforesaid QE-EUS to CEH-EUS. The authors concluded that the diagnostic accuracy of QE-EUS in pancreatic masses is superior to CEH-EUS and, furthermore, that addition of CEH-EUS does not significantly increase the diagnostic accuracy of QE-EUS.

THE ROLE OF EUS IN STAGING OF PANCREATIC CANCER

Surgery is the only curative treatment for pancreatic cancer. Statistics for survival in pancreatic cancer, where 5 year survival rates are as low as 10%-25% after a successful surgery[66,67], have been changing because of identification of appropriate candidates for surgery by a good staging, approaching a 5 year survival rate of 40% if margins and nodes are negative and the resection is made by experienced surgeons[68,69].

However, even with the newest diagnostic workup, pancreatic cancer at laparotomy is often found to be more advanced than originally thought[70,71].

Currently, the preferred modality for pancreatic can-

cer staging and assessing resectability is CT because its low cost and high availability[72] and MRI for preoperative assessment of pancreatic cancer, with an accuracy of 86% *vs* 71% even with comparable sensitivity of MRI for detecting pancreatic cancer (88%-96%)[73].

EUS has been found to be superior to the recent multidetector CT (MDCT) for T staging[74-77], with less risk of overstaying in comparison to both CT and MRI[78] so that patients are not being ruled out of a potentially beneficial resection. In a recent study, the sensitivity of EUS was higher than MDCT but MDCT was more specific, especially in the assessment of vascular invasion. The correct decision could be achieved in 63% in patients with either MDCT or EUS, in 9% of patients with EUS alone and in 14% of patients with MDCT alone, but the success rate rises to 86% when they are used in combination[79].

The accuracy for N staging with EUS is 64%-82%[80]. Only one study found that EUS is also better than CT for N-staging (93.1% *vs* 87.5% respectively), but most of the studies have found no difference between CT and EUS in predicting resectability in relation to node involvement[74,78-81]. Criteria for the identification of lymph node metastasis are used in different studies: spherical shape, hypoechoic node, well delineated boundaries and 10 mm diameter or more. These criteria normally are not enough and EUS-FNA is often required.

EUS has been found to be better at peripancreatic and periceliac lymphadenopathy detection (87.5%), and vascular infiltration (90%), especially for mesenteric vessels that also have a higher ability to correctly predict surgical resectability[82-84]. EUS has shown a good ability to detect vascular invasion, showing low sensitivity in the superior mesenteric artery (17%) and celiac artery (50%), although the portal venous system was correctly assessed by EUS in 95% of cases, compared with angiography (85%) and CT (75%)[85,86]. However, differently from radial EUS, linear EUS can show arterial vessels longitudinally using a linear image and both the celiac and superior mesenteric arteries are easily followed from the stomach. A recent prospective study by Tellez-Avila *et al*[87], in which the accuracy of linear-EUS and CT to determinate vascular invasion is evaluated in 50 patients with pancreatic cancer, EUS is a very good option to detect vascular invasion and is especially sensitive for arterial invasion (PPV EUS 100% *vs* PPV CT 60%).

Tumor conditions may also affect the accuracy of EUS staging[88], such as peritumoral inflammatory changes and attenuation of ultrasound beam in large tumors. For this reason, tumors smaller than 3 cm in size are more accurately staged with EUS.

THE ROLE OF EUS AS PALLIATIVE TREATMENT OF PANCREATIC CANCER: THERAPEUTIC OPTIONS

In patients with advanced unresectable disease, chemotherapy, radiation or a combination of both may positively influence overall survival and quality of life. The

therapeutic spectrum of EUS has turned endoscopy into an integral component of palliative treatment in patients with inoperable disease. EUS offers access to lesions in different parts of the pancreas, including anatomical regions that are difficult to approach percutaneously.

CELIAC PLEXUS NEUROLYSIS

Pain is one of the most prevalent symptoms in pancreatic cancer at presentation (75%) and its incidence increases as the disease advances to more than 90% of patients[89]. Pain control is the main therapeutic goal for clinicians in palliative care of pancreatic cancer patients and the conventional management with high doses of narcotics and the inherent adverse effects may further impair quality of life[90-92].

Before 2010, celiac plexus neurolysis (CPN) was considered an effective technique for controlling pain and reducing narcotic requirements in patients with pancreatic cancer[89-93]. However, a recent meta-analysis of five randomised controlled trials documented a fair response to CPN with an overall reduction in the visual analog pain scores[89]. A recent systematic review that aimed to determinate its efficacy and safety in reducing pancreatic pain found that the statistical evidence of the superiority of CPN over analgesic therapy or reducing opioid use was weak[94,95]. On the other hand, a recent randomised trial of early EUS guided CPN concluded that early EUS-CPN provides better pain relief in patients with painful, inoperable pancreatic adenocarcinoma and may prevent progressive increases in morphine consumption compared with conventional management, especially in patients who do not receive chemotherapy and/or radiation therapy, so they recommend it to be considered during diagnostic and staging EUS in all patients with predicted survival of several months where a confirmation of painful, locoregional and inoperable pancreatic cancer is obtained[95]. Despite better pain control, early EUS-CPN did not produce a demonstrable improvement in quality of life, but this was not a study powered to look for effects on quality of life.

BILIARY DRAINAGE

EUS-guided biliary drainage (EUS-BD) has been described as an alternative method to achieve internal biliary drainage in those patients in whom ERCP is not feasible. EUS-guided cholangiopancreatography (ESCP) was first described by Wiersema *et al*[96] in 1996. ESCP using either direct access or a rendezvous technique has shown a technical success between 75%-100%[97-100], although complications can reach up to 20%, especially in the early phase of the learning curve of the procedure[101].

ESCP can be performed through different routes (transgastric, transduodenal) and with different techniques (rendez-vous, hepaticogastrostomy, choledocoduodenostomy)[102]. In the rendezvous technique, the bile duct is punctured with a 19 or 22 G needle under EUS guidance and a wire is antegradely guided through any

stricture and across the papilla under fluoroscopic guidance. The echoendoscope is then removed, leaving the wire in place, and the procedure is completed with a duodenoscope.

In hepaticogastrostomy and choledocoduodenostomy, the bile duct is punctured, preferably with a 19 G needle, a wire is guided into the bile duct and, after dilation of the transmural tract, a plastic or metallic stent is inserted.

EUS-GUIDED RADIOFREQUENCY ABLATION

EUS-guided radiofrequency ablation (EUS-RFA) has been successfully tested in two porcine studies for ablation of both lymph nodes[103] and the pancreas [104]. RFA was performed with a EUS adapted probe which was inserted through the lumen of a FNA needle. At histological analysis, the ablation effect was limited to the lesions and a direct correlation was seen between probe length and length and diameter of the necrosis.

EUS-FNI FOR TUMOR ABLATION AND INTRATUMORAL DRUG DELIVERY

EUS-FNI has made the intratumoral delivery of ethanol, chemotherapy as paclitaxel[105] or biological agents[106] possible in a precise real time tumor visualisation. Several studies have proved that it is a promising and safe technique, but validation in larger studies over longer follow-up periods is necessary.

EUS GUIDED RADIATION THERAPY

In a recent study with 22 patients with pancreatic cancer in which an average of 10 radioactive iodine-125 seeds were implanted under EUS guidance, the authors noticed a decrease in pain during the week following brachytherapy but there was no long-term survival benefit [107]. Recent reports concluded that EUS is safe for fiducial placement in pancreatic tumors [107] and for submucosal injection of tantalum for identification of the tumor during radiation and surgery[108].

In conclusion, EUS plays an important role in the diagnosis of pancreatic cancer, including FNA with cytological or histological confirmation. Staging of pancreatic cancer is crucial and CT and EUS are the cornerstones of staging, currently providing the more accurate results. Furthermore, EUS also has a therapeutic role, providing biliary drainage when it is not feasible with ERCP and pain relief. EUS can also have future applications on pancreatic cancer management.

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2014 ADVANCES IN PANCREATIC CANCER

Metastatic tumors to the pancreas: The role of surgery

Cosimo Sperti, Lucia Moletta, Giuseppe Patanè

Cosimo Sperti, Lucia Moletta, Giuseppe Patanè, Department of Surgery, Oncology and Gastroenterology, 3rd Surgical Clinic, University of Padua, 35128 Padova, Italy

Author contributions: Sperti C and Moletta L conceived the article and drafted the manuscript; Patanè G carried out literature review and preparation of the manuscript; all authors read and approved the final manuscript.

Correspondence to: Cosimo Sperti, MD, Department of Surgery, Oncology and Gastroenterology, 3rd Surgical Clinic, University of Padua, via Giustiniani 2, 35128 Padova, Italy. csperti@libero.it

Telephone: +39-049-8218845   Fax: +39-049-8218821

Abstract

Pancreatic metastases from other primary malignancies are a rare entity. By far, the most common primary cancer site resulting in an isolated pancreatic metastasis is the kidney, followed by colorectal cancer, melanoma, breast cancer, lung carcinoma and sarcoma. Only few data on the surgical outcome of pancreatic resections performed for metastases from other primary tumor have been published, and there are no guidelines to address the surgical treatment for these patients. In this study, we performed a review of the published literature, focusing on the early and long-term results of surgery for the most frequent primary tumors metastasizing to the pancreas. Results for the Literature's analysis show that in last years an increasing number of surgical resections have been performed in selected patients with limited pancreatic disease. Pancreatic resection for metastatic disease can be performed with acceptable mortality and morbidity rates. The usefulness of pancreatic resection is mainly linked to the biology of the primary tumor metastasizing to the pancreas. The benefit of metastasectomy in terms of patient survival has been observed for metastases from renal cell cancer, while for other primary tumors, such as lung and breast cancers, the role of surgery is mainly palliative.

Core tip: Pancreatic metastases represent a rare but increasing entity among pancreatic tumors. We have reviewed the literature's reports of the more common metastatic tumors to the pancreas, evaluating early and long-term results of surgery. Pancreatic resection may appear a safe and feasible option also in metastatic tumors, but long term survival is achieved substantially only in renal cell cancer. In other metastatic tumors, pancreatectomy may offer a good palliation in selected patients, but it is to remark that surgery is only one option in the multimodality treatment of metastatic disease to the pancreas.

INTRODUCTION

Pancreatic metastases from other primary cancers are rare[1]. Approximately 2% of pancreatic cancers are metastatic from other primary site[2,3]. In different autopsy series, a wide range of malignant tumors have been found to metastasize to the pancreas and the most frequent primary locations of tumor were the kidney, breast, colon, skin and lung[4-6]. It may be difficult to differentiate a pancreatic metastasis from a primary pancreatic tumor, being the clinical presentation and the radiological characteristics similar for both primary and secondary neoplasms[7,8]. Pancreatic metastases are asymptomatic in more than 50% of cases: they are often detected during follow-up
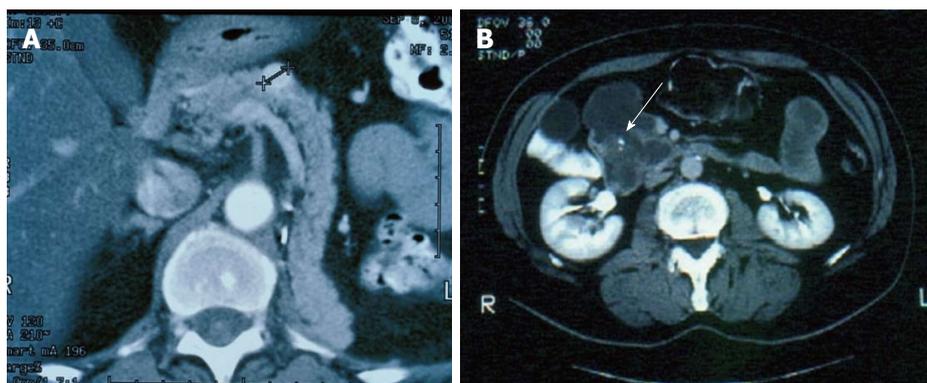
Figure 1 Computed tomography scan of the abdomen. A: Computed tomography (CT) scan of the abdomen showing a contrast-enhanced pancreatic metastasis from a renal cell carcinoma; B: CT scan of the abdomen showing an hypodense metastatic lesion of the pancreatic head from a colon carcinoma.
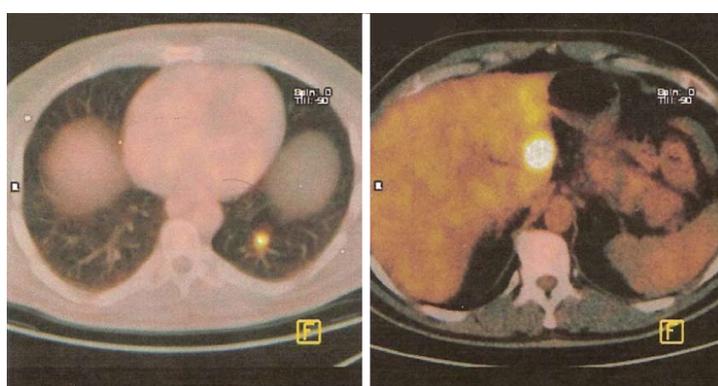
Figure 2 Positron emission tomography/computed tomography imaging showing a pathologic uptake of the tracer in the region of the pancreatic neck and in the left lung from a melanoma.

investigations after surgery for a primary lesion or as an incidental finding on imaging studies performed for an unrelated condition[9,10]. At CT scan, pancreatic metastases may appear as hypervascular lesions, like in renal cell cancer (RCC) metastases (Figure 1A) or, as in the case of colon and melanoma metastases, as hypodense masses (Figure 1B). Positron emission tomography may be helpful in order to exclude other metachronous lesions than the pancreatic one or other primary synchronous tumors (Figure 2).

Pancreatic metastases occur in two different clinicopathological settings, either as one manifestation in widespread disease or as an isolated mass of the pancreas. However, only few patients present with a single potentially resectable pancreatic lesion[11] and the most common presentation is that of a widespread metastatic disease[12]. The number of pancreatic resections for metastatic lesions in high volume centers has gradually increased, probably because of the greater knowledge of these clinical entities and the greater availability of radiological studies in asymptomatic patients[13]. In recent years, different studies showed an improved survival in patients undergoing lung or liver resection for metastatic lesions from colorectal cancer[14,15]. Pancreatic resections were for many years associated with high rates of morbidity and mortality, but recent data have clearly shown that pancreatic surgery is safe and feasible in high-volume clinical centers: the lower morbidity and mortality rates make pancreatic resection an acceptable indication also in

case of metastatic lesions[16-18].

In this study, we have reviewed the literature's reports of the more common metastatic tumors to the pancreas, evaluating early and long-term results of surgery.

RESEARCH

The published Literature was systematically searched using PubMed and free text search engines up to October 2013. Search terms included: pancreatic neoplasms/secondary, pancreatectomy, renal cell cancer, breast cancer, melanoma, colorectal cancer, sarcoma, lung cell cancer. The "related articles" function was used to broaden the search and all abstracts, studies, and citations retrieved were reviewed. Only articles published in the English language, with abstracts, and human studies only were selected. Case reports were included for the less common neoplasms. In the case of sequential publications, the report with the most comprehensive information regarding the study population was selected. Studies were excluded from the analysis if: (1) the outcome and parameters of interest were not clearly reported, and (2) it was impossible to extract the data from the published results. Two investigators (LM and GP) reviewed the titles and abstracts and assessed the full text of the articles obtained to establish eligibility. The following data were extracted from each study: first Author, year of publication, number of patients, perioperative morbidity and mortality, and long-term outcome. For statistical analysis, overall

averages are presented as weighted means (range) unless otherwise stated.

The preliminary literature search showed 1536 studies matching the initial search criteria. After screening, 108 studies evaluating metastases to the pancreas were selected. There were 41 case series (more than two patients) and 67 single case reports, for a total of 418 patients with secondary tumor of the pancreas: metastases were mainly from RCC (*n* = 293), followed by melanoma (*n* = 38), colorectal cancer (*n* = 37), breast cancer (*n* = 19), sarcoma (*n* = 18), and lung cancer (*n* = 13).

The results of the Literature's review are showed for each tumor considered.

RCC

By far, the most common primary cancer site resulting in an isolated pancreatic metastasis is the kidney. RCC accounts for approximately 2% of all adult malignancies. Among kidney-limited diseases, RCC has a high overall survival rate (up to 95%)[19]. However 20% to 30% of patients have metastases at presentation, and the 5-year survival rate is less than 10% once metastases spread[20]. In autopsy series in primary RCC, pancreatic metastases were noted in 1.3% to 1.9%[21]. Hirota *et al*[22] revealed that a characteristic of the patients in this group was the long disease-free interval from the time of the nephrectomy to the diagnosis of metastatic disease. This long disease free interval indicates a biological pattern of slow growth, favouring local surgical resection. Pancreatic metastases are often the only metastatic lesions and they seems related to a good prognosis[17,23]. Pancreatic metastases are only rarely symptomatic; therefore a long follow-up (> 10 years) is indicated in patients with RCC[10]. At CT scans metastases from RCC appear as hypervascular lesions, and a differential diagnosis must be done with primary endocrine tumors[24]. OctreoScan® scintigraphy is not always able to differentiate neuroendocrine lesions from pancreatic metastases from RCC. A recent study on metastatic RCC showed the presence of positive scintigraphy, and thus the presence of somatostatin receptors, in 9 of 11 cases[25]. A percutaneous fine-needle biopsy to confirm the clinical suspicion is seldom necessary. Pancreatic metastases from RCC can occur a long time after the diagnosis of the primary RCC. The presence of synchronous pancreatic lesions is less frequent (15%-27% of cases)[22,26,27] and it may be an expression of a widespread disease, thus limiting the benefit of a pancreatic metastasectomy. In a recent review by Masetti *et al*[28], univariate analysis showed that a disease-free survival time less than 2 years in metachronous metastases was associated with a worse survival. The detection of multiple pancreatic metastases occurs more often in RCC than in other primary malignancies and this must be taken into account in the planning of the surgical treatment of these patients[23]. In a review of the literature we found 29 studies reporting on pancreatic resection for metastatic RCC (Table 1, [3,9,10,12,16,23,24,28-49]). Only reports with detailed clinical and follow-up informations on 2 or more patients were se-

lected, while single case-reports were excluded. Informations on 293 patients have been published. Among these, the median interval between nephrectomy and pancreatic recurrence was 104 mo (range 0-348 mo). Perioperative mortality occurred in only 4 patients with a mortality rate of 1.5%. Morbidity was difficult to assess because this information wasn't always reported and because in many reports it wasn't possible to differentiate morbidity rate after resection for RCC from other primary tumors. Among the available data, the overall morbidity rate was 13.3%. Median follow-up was 36.8 mo (range 3-130 mo). Eighty patients died and among them 56 patients died of recurrent disease (in some reports this information was not available). Tanis *et al*[34], in a recent review of 421 patients undergoing resection of pancreatic RCC metastases, reported an actuarial 5 years survival rate, calculated on 321 patients for which data were available, of 72.6% and the survival of these patients was compared to that of 73 non-surgically treated patients: 2 and 5 years overall survival rates were 80% and 72% in the operated group and 41% and 14% in the non-operated group. Bassi *et al*[17] reported in a single-centre series a great 5-year survival benefit after surgical resection compared with conservative treatment of unresectable disease (53% *vs* 26%). Pancreatic metastases from RCC are reported to have a better prognosis when compared to other primary tumors, therefore an aggressive treatment, *i.e.*, surgical resection, should be considered in these patients. Reddy *et al*[9] demonstrated that the median survival for pancreatic metastases from RCC was 4.8 years *vs* 0.9 years for metastases from melanoma. Konstantinidis *et al*[12] reported a 5-year actuarial survival of 61%, and they demonstrated that RCC patients had a better median survival (8.7 years) compared to other pathologies. Chemotherapy, immunotherapy, and radiotherapy have generally proved to be ineffective for primary RCC or metastatic disease. Despite promising results with immunotherapy using IL-2, a complete response occurred in less than 15% and was rarely durable[50,51]. In more recent years several angiogenetic agents (bevacizumab, sunitinib, sorafenib) have showed promising results[52]. Therefore a multidisciplinary approach has to be recommended in the treatment of pancreatic metastates from RCC and further studies are needed to establish the way to combine surgery with medical treatment in the different periods of the disease.

Colorectal cancer

In the English Literature only few studies on pancreatic resection for metastatic colorectal cancers have been published so far[53], representing only single case reports, rarely more than two patients[54]. In recent years, several studies demonstrated encouraging results on surgical resections for metastatic colorectal cancer to the liver and lung; on the other hand, only few data are available for pancreatectomies in metastatic colorectal cancer[55]. In a review of the literature, we selected 24 studies regarding surgical treatment of pancreatic metastases from colorectal cancer (Table 2[9,24,26,29,37,54-72]). Informations on

Table 1 Pancreatic resections for metastates from renal cell carcinoma

Ref.	No. of patients	Treatment	Mortality-morbidity	Follow-up; (mo) median (range)	Dead
Niess *et al*[29]	16	DP (10); PPPD (3); PD (2); TP	0-NA	39 (4-76)	6 (37.5%)
Yazbek *et al*[30]	11	NA	5/11/2001	78 (12-108)	4 (36.3%)
Alzahrani *et al*[31]	12 (7 resected)	DP (3); TP (2); CP; PD	1/7/2000	19 (1-96)	5 (41.6%)
D'Ambra *et al*[32]	8 (7 resected)	NA	0-3/7	43 (12.9-74.5)	NA
You *et al*[33]	7	NA	0-NA	34 (7-69)	1 (14.3%)
Konstantinidis *et al*[12]	20	NA	0-NA	36.8 (0.5-143)	NA
Masetti *et al*[28]	6	TP (5); PD	1/6/2000	3	0
Tanis *et al*[34]	10	NA	0-NA	NA	3 (30%)
Zerbi *et al*[10]	36 (23 resected)	DP (11); enucleation (5); PD (4); TP (2); CP	0-14/23	31 (12-98)	9 (25%)
Reddy *et al*[9]	21	NA	0-NA	57.6 (4.2-219.6)	19 (90.5%)
Schauer *et al*[35]	10	TP (5); PD (3); PPPD; DP	2/10/2001	56 (56-60)	NA
Karimi *et al*[36]	3	DP (3)	NA	96 (60-156)	0
Eidt *et al*[37]	7	PPPD (4); TP (2); DP	0-NA	36 (12-156)	2 (28.6%)
Sellner *et al*[23]	3	NA	0-NA	48 (36-60)	0
Crippa *et al*[24]	5	DP (3); PPPD; PD	0-NA	41 (21-95)	1 (20.0%)
Wente *et al*[38]	15	DP (7); PD (3); TP (3); PP (2)	4/15/2000	10 (1-28)	1 (6.7%)
Jarufe *et al*[39]	7	NA	1-NA	24	NA
Moussa *et al*[40]	10 (7 resected)	PD (6); TP	1-NA	61	6 (60.0%)
Law *et al*[41]	14	NA	0-NA	130 (32-315)	3 (21.4%)
Sperti *et al*[16]	2	TP; CP + enucleation	0-NA	18 (14-21)	1 (50.0%)
Zacharoulis *et al*[42]	3 (2 resected)	NA	2/3/2000	26 (7-88)	0
Yachida *et al*[43]	5	NA	0-NA	12 (2-160)	0
Faure *et al*[44]	8	PD (5); TP (3)	1/8/2000	38 (13-83)	2 (25.0%)
Sohn *et al*[45]	10	PPPD (5); DP (2); PD (2); TP	0-3	8 (3-117)	2 (20.0%)
Ghavamian *et al*[46]	11	DP (8); TP (3)	0-NA	50 (5-120)	3 (27.3%)
Kassabian *et al*[47]	5	CP; PPPD; TP; PD; DP	0-NA	48	1 (20.0%)
Thompson *et al*[48]	21 (15 resected)	DP (9); PP (4); PD (2)	0-NA	NA	NA
Butturini *et al*[49]	5	NA	NA	19 (7-27)	1 (20.0%)
Z'graggen *et al*[3]	2	TP (2)	0-NA	20 (20-40)	2 (100%)
Total	293 (270 resected)	DP (59); TP (32); PD (31); PPPD (16); CP (3); PP (6); enucleation (6)	4 (1.5%)-36 (13.3%)	36.8	72/227 (31.7%)

NA: Not available; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectony; DP: Distal pancreatectomy; CP: Central pancreatectomy; TP: Total pancreatectomy; PP: Partial pancreatectomy.

37 patients were available, 24 with a primary neoplasm of the colon and 11 with a primary rectal cancer. Among these patients, 28 presented with a single pancreatic metastasis and in 9 cases an associated surgical procedure was required for metastatic disease in other sites. There was no perioperative mortality. After pancreatic resection, a recurrence of disease occurred in 19 patients, with a median survival time of 21 mo (range 5-105 mo). Sixteen patients are alive with a median survival time of 12 mo (range 1.5-43 mo), while 5 patients are alive with recurrent disease (6 to 43 mo). It is interesting to note that all patients experienced a relief of symptoms (abdominal pain and obstructive jaundice) after surgical resection of metastases and they remained asymptomatic until recurrence of the disease. It is impossible to establish whether the same results can be achieved in these patients with a more conservative treatment, such as chemotherapy, because of the lack of information regarding the outcome of patients undergoing pancreatic resection and patients undergoing only chemotherapy. Considering the data available in the literature, it seems reasonable to consider surgery for pancreatic metastases from colorectal cancer a palliative treatment. However, it has to be remark that a multidisciplinary approach has to be recommended in

the treatment of pancreatic metastates from colorectal cancer, and an aggressive surgical approach may be considered in selected cases, in particular in symptomatic patients with isolated pancreatic metastasis.

Melanoma

Metastases from malignant melanoma can be located in the gastrointestinal tract (50%-60% of cases of malignant melanoma in autopsy series), although the clinical diagnosis occur in only 1.5% to 4.4% of patients[73]. A few cases of long-term survival after radical surgical resection of melanoma metastases in the gastrointestinal tract have been reported[74,75], but the role of surgery in the treatment of pancreatic metastases from melanoma is unknown, due to the lack of data regarding these clinical entities[9,76]. When compared to other primary tumors metastasizing to the pancreas, melanoma seems related to a poor prognosis[28]. In a literature review, we collected a total of 23 reports (19 single-patient reports, 1 with two patients, 3 with more than 2 patients) on surgical treatment of pancreatic metastases from melanoma (Table 3[24,37,58,74,77-95]). Among these patients, 12 had a primary skin melanoma, 6 had an ocular melanoma, 1 had a melanoma of the nasal cavity and in 19 cases the primary

Table 2 Pancreatic resections for metastatic colorectal cancer

Ref.	Year	No	Site of primary	Interval (mo)	Treatment	Survival (mo)	
						Dead	**Alive**
Roland *et al*[56]	1989	1	Colon	NR	DP		27, AWD
Nakeeb *et al*[57]	1995	1	Colon	34	PD		43, AWD
Harrison *et al*[58]	1997	2	Colon	15	PD	41	
			Colon	15	PD	21	
Inagaki *et al*[59]	1998	1	Rectum	132	DP		8
Yoshimi *et al*[60]	1999	1	Colon	51	PD	24	
Le Borgne *et al*[26]	2000	1	Colon	60	PD	12	
Tutton *et al*[61]	2001	1	Colon	23	DP		12
Torres-Villalobos *et al*[62]	2004	1	Cecum	8	DP		6
Crippa *et al*[24]	2006	1	Colon	7	PPPD	13	
Matsubara *et al*[55]	2007	1	Rectum	28	PD	24	
Eidt *et al*[37]	2007	1	Colon	12	PPPD	105	
Shimoda *et al*[63]	2007	1	Rectum	44	PD	8	
Bachmann *et al*[64]	2007	2	Rectum	24	DP		1.5
			Rectum	30	DP		6
Sperti *et al*[54]	2008	9	Colon (7)	10-80	PD (2)	525	30, AWD
			Rectum (2)		PPPD (3)		
					DP (4)		
Reddy *et al*[9]	2008	2	NR	NR	NR		42
Grève *et al*[65]	2008	1	Rectum	54	DP	NR	NR
Gravalos *et al*[66]	2008	1	Cecum	17	DP		12
Machado *et al*[67]	2010	1	Colon	105	DP	9	
Lasithiotakis *et al*[68]	2010	1	Colon	24	PD	27	
Lee *et al*[69]	2010	1	Rectum	24	DP		12
Stoltz *et al*[70]	2011	1	Colon	24	DP		6, AWD
Georgakarakos *et al*[71]	2011	1	Colon	12	PD		6
Tanemura *et al*[72]	2012	2	Rectum	72	MSPP		16
			Rectum	84	DP		6
Niess *et al*[29]	2013	2	Colon	0	PPPD	68	21, AWD
			Colon	14	DP		
Total		37	Colon (24)	24	PD (11)	21	12
			Rectum (11)	(median)	DP (17)	(median)	(median)
			NR (2)		PPPD (6)		
					MSPP (1)		

NR: Not reported; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; MSPP: Middle-segment-preserving pancreatectomy; SMV: Superior mesenteric vein; AWD: Alive with disease.

site of melanoma was unknown. No perioperative mortality was reported. Twenty patients died of recurrent disease: the median survival time of these patients was 10 mo (range 3-25 mo). Thirteen patients are alive at 6 to 108 mo (median 16 mo); 2 patients were alive, with recurrence, at 8 and 12 mo respectively. Although malignant melanoma is associated with a poor prognosis and the role of surgery seems limited to palliation, some cases of a prolonged survival after surgical removal of melanoma metastases have been reported[74] and, when possible, surgical resection seems to be the most effective therapeutic option available today[96,97]. However, there are no sufficient data in the literature to compare patients treated with only conservative management (chemotherapy) with surgical resected patients. Therefore surgical resection for pancreatic metastases from melanoma should be considered a palliative treatment, to be taken in account in pancreatic isolated lesions as a part of the multimodality treatment of this clinical entity.

Breast carcinoma

Pancreatic metastases from breast cancer are rare, with a reported rate of 13% in an autopsy series[98]. Metastatic breast cancer is usually a widespread disease, with isolated pancreatic lesions being an occasional event. In a literature review, we selected 16 studies regarding patients undergoing surgery for pancreatic metastases from breast cancer (Table 4)[9,24,26,40,57,99-110]. Breast cancer that metastasize to the pancreas may have a long latency period between the primary tumor diagnosis and the metastasis occurrence (median 39.5 mo, range 0-216). Solitary pancreatic metastasis was present in 17 patients, and 1 underwent also a subtotal gastrectomy for extrapancreatic involvement. There was no perioperative mortality. Five patients died of recurrent disease: the survival time was available in only three of these patients and the median was 26 mo (range 7-36 mo). Fourteen patients are alive at 5 to 80 mo (median 19), although 5 patients had a short follow-up (up to 12 mo) and in one patients follow-up time is not reported; 3 patients were alive, with recurrence, at 11 to 48 mo. All patients experienced a relief of symptoms (abdominal pain and obstructive jaundice) after surgical resection of metastases and they remained asymptomatic until recurrence of the disease. Masetti *et al*[28]

Table 3 Pancreatic resections for metastatic melanoma

Ref.	Year	No	Interval (Yr)	Primary site	Surgery	Follow-up (mo)	Outcome
Dasgupta *et al*[77]	1964	1	2	Skin	DP + duodenal resection	10	DOD
Johansson *et al*[78]	1970	1	12	Ocular	PD	11	ANED
Lasser *et al*[79]	1990	1	8	Skin	PD	10	ANED
Bianca *et al*[80]	1991	1	NA	NA	PD	12	AWD
Brodish *et al*[81]	1993	1	34	Skin	DP	8	AWD
Harrison *et al*[58]	1997	1	NR	NA	PD	108	ANED
Medina-Franco *et al*[82]	1999	1	NA	NA	PPPD	6	DOD
Wood *et al*[74]	2001	8	NA	NA	PD	37.5%[1]	DOD
Hiotis *et al*[83]	2002	1	NR	NR	PD	NR	DOD
Camp *et al*[84]	2002	1	6	Ocular	DP	20	ANED
Nikfarjam *et al*[85]	2003	2	12, 13	Ocular	PPPD, TP	6, 7	ANED
Carboni *et al*[86]	2004	1	9	Skin	PD	4	DOD
Crippa *et al*[24]	2006	1	2.8	Skin	PPPD	14	DOD
Belágyi *et al*[87]	2006	1	6	Skin	Enucleation	4	DOD
Edit *et al*[37]	2007	4	3, 4, 4, 14	NA	PPPD (4)	12, 25	DOD
						30, 76	ANED
Vagefi *et al*[88]	2009	1	28	Ocular	DP	NR	NR
Sperti *et al*[89]	2009	1	3	NA	DP	24	DOD
He *et al*[90]	2010	1	5	Ocular	DP	25	ANED
Lanitis *et al*[91]	2010	1	5	Skin	PD	96	ANED
Moszkowicz *et al*[92]	2011	1	15	Skin	PD	NA	NA
Portale *et al*[93]	2011	1	7	Skin	DP	NA	ANED
Goyal *et al*[94]	2012	5	3, 22, ?, 5, ?	Skin (3), NA (2)	PPPD (4),DP(1)	15, 3, 11.4, 4.5, 25	DOD
Sugimoto *et al*[95]	2013	1	1	Nasal	DP	10	DOD
Total		38	6 (median)	Skin = 12; Ocular = 6; Nasal = 1; NA = 18; NR = 1	PD (16), DP (9), PPPD (11), TP (1), Enucleation (1)	11, 7 (median)	

[1]5 years survival rate. NR: Not reported; NA: Not available; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; MSPP: Middle-segment-preserving pancreatectomy; SMV: Superior mesenteric vein; DOD: Dead of disease; ANED: Alive not evidence of disease; AWD: Alive with disease.

analysing the prognostic factors in metastatic tumors to the pancreas, found at univariate survival analysis a 2-years probability of survival of 57.1% in pancreas metastases from breast cancer and a 5-years probability of survival of 34.3%. Even in the case of pancreatic metastases from breast cancer it is impossible to establish the course of the disease without surgical resection and to assess the real benefit in survival after metastasectomy. However, in selected patients with limited pancreatic disease, surgical resection could have a palliative role in association with chemotherapy, hormonal therapy and radiation therapy in the multimodality treatment of metastatic breast carcinoma.

Lung cancer

Lung cancer metastasize to many site, but most frequently to bone, liver and adrenal glands[111,112]. Isolated pancreatic metastases from lung cancer are extremely rare[76] and they are usually metachronous lesions, identified at follow-up investigation. The few reports available in the literature show that small cell lung cancer (SCLC) represents the most typical histological subtype metastasizing to the pancreas[113].

The usefulness of surgical resection for pancreatic metastasis from lung cancer is difficult to assess because of the rarity of this type of lesion. Additionally, most cases of pancreatic metastasis from lung cancer are unresectable at the time of diagnosis because the disease is already widespread. Z'graggen *et al*[3] and Moussa *et al*[40] reported four patients each with secondary metastasis from lung cancer (including small cell lung cancer): there were no resectable cases mainly due to local invasion and metastases to other organs. Hiotis *et al*[83] reported three cases of pancreatic resections for metastatic lung cancer, with a poor long-term survival after surgery. In a recent review of the literature, Reddy *et al*[9] reported pancreatic resections from lung cancer as having the worst outcome when compared to other primary tumors type metastatic to the pancreas. In a literature review, we selected 12 studies reporting surgical resection for pancreatic involvement from lung cancer (Table 5[24,26,57,68,82,83,102,114-118]). Among these patients, in 10 cases the primary lung cancer was a NSCLC, 1 case was a SCLC and in the last patient the primary lung cancer is not specified. One patient died after surgical resection. Five patients died of recurrent disease, with a median survival time of 7 mo (range 3-14 mo). Six patients are alive with a median survival time of 19 mo (range 6-24 mo). In all cases, preoperative symptoms (obstructive jaundice and abdominal pain) disappeared after surgery. Pancreatic metastases from lung cancer have a poor prognosis and treatment options for metastatic lung cancer lesions to the pancreas are mainly

Table 4 Pancreatic resections for metastatic breast cancer

Ref.	Yr	No	Interval (mo)	Treatment	Survival (mo)	
					Dead	**Alive**
Bednar *et al*[99]	2013	1	216	PD		48 mo, AWD
Razzetta *et al*[100]	2011	1	0	PD		11 mo, AWD
Bonapasta *et al*[101]	2010	1	23	PD	36	
Mourra *et al*[102]	2010	1	9	DP		20 mo
Sweeney *et al*[103]	2009	1	60	DP		NA
Reddy *et al*[9]	2008	1	NR	NR	NR	13 mo
Jiménez-Heffernan *et al*[104]	2006	1	0	PD		10 mo
Tohnosu *et al*[105]	2006	1	52	DP		5 mo
Crippa *et al*[24]	2004	3	60/36/84	PPPD (3)	26	21AWD/37
Moussa *et al*[40]	2004	1	45	TP	7	
Minni *et al*[106]	2004	1	26	enucleation		80
Ogino *et al*[107]	2003	1	72	PD	Dead (-)	
Le Borgne *et al*[26]	2000	1	0	PD		12
Nomizu *et al*[108]	1999	1	80	PD		18
Mehta *et al*[109]	1997	1	36	PD		27
Nakeeb *et al*[57]	1995	1	19	PD		12
Azzarelli *et al*[110]	1982	1	43	PD		72
Total		19	39.5 mo (median)	PD (10) DP (3) PPPD (3) Enucleation (1) TP (1)	26 (median)	19 (median)

NR: Not reported; DP: Distal pancreatectomy; NA: Not available; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; TP: Total pancreatectomy; AWD: Alive with disease.

palliative.

Sarcoma

Metastatic sarcoma has generally a poor survival, and radical surgical represent the only therapeutical chance for these patients. Isolated pancreatic involvement by sarcomas is rarely encountered: in a recent experience Yoon *et al*[119] reported only 2 cases (4%) of sarcomas among 53 patients with pancreatic metastases collected at their Institution. So, the outcomes for patients with metastatic sarcoma who did or did not pancreatic resection are unknown[53]. In their review, Reddy *et al*[53] collected only 10 patients with isolated pancreatic metastasis with a median survival of 40 mo and 5-year survival of 14%. Even if pancreatic metastases from sarcoma seem related with a modest survival, the few data available does not allow to draw any definitive conclusion. Recently, Robert *et al*[120] reported a case of leiomyosarcoma metastatic to the pancreas and collected 17 of the such cases published in the Literature. Clinical details were available in only 8 reports, and 7 patients underwent pancreatic resection: 5 patients were alive (one with disease) and 2 died, with a median survival time of 23 mo. As for other cancers, resection of pancreatic metastases from sarcoma is substantially justified in individual basis.

In recent years, an increased number of surgical resections for pancreatic metastases has been performed in high-volume centers. It seems reasonable that resection is indicated for an isolated and resectable metastasis in a patient fit to tolerate pancreatectomy, evaluating each single case on an individual basis and with a multidisciplinary approach.

The type of surgical procedure is another controversial aspect in pancreatic metastases. Standardized pancreatic resection adapted to the location of the tumor, in terms of partial pancreaticoduodencectomy, distal pancreatectomy, and total pancreatectomy, is generally recommended for the management of isolated pancreatic metastases. Bassi *et al*[17] observed a high rate of pancreatic recurrences after atypical resections and recommended standard radical resection. Considering the high frequency of multiple metastases, a recurrence after surgical resection could be related to multifocality of the tumor rather than to an atypical surgical procedure[18]. Since pancreatic metastases is often multifocal, partial pancreatectomies require thorough exploration of the pancreatic remnant by palpation and ultrasound. Intraoperative ultrasound is a very useful device: it guides the surgeon in choosing the most appropriate surgical procedure by defining the presence of multiple pancreatic lesions and the proximity of the metastasis to the Wirsung duct[18]. Surgical strategy should be tailored on each single case, in order to achieve an R0 resection and ensuring the absence of further disease in the pancreatic parenchyma. Surgical resection may be considered also in selected cases of extrapancreatic disease, if technically feasible[16]. The effectiveness of resection for pancreatic metastases is mainly dependent on the tumor biology of the primary cancer.

The benefit of metastasectomy in terms of patient survival has been observed for metastases from RCC, while for other primary tumors the role of surgery is mainly palliative. Patients with pancreatic metastases

Table 5 Pancreatic resections for metastatic lung cancer						
Ref.	No	Interval (mo)	Type of primary	Treatment	Survival (mo)	
					Dead	Alive
Igai *et al*[114]	1	60	NSCLC	PD		6
Lasithiotakis *et al*[68]	1	6	NSCLC	PD	/	/
Mourra *et al*[102]	2	0, 10	NSCLC	DP (2)	10	20
Wilson *et al*[115]	1	NA	NSCLC	PD		22
Mori *et al*[116]	1	22	NSCLC	PPPD		24
Pericleous *et al*[117]	1	0	NSCLC	PPPD		18
Crippa *et al*[24]	1	5	NSCLC	PPPD	14	
García Vidal *et al*[118]	1	0	NSCLC	PD		NA
Hiotis *et al*[83]	1	NA	NA	DP	DOD	
Le Borgne *et al*[26]	1	0	SCLC	PD	4	
Medina-Franco *et al*[82]	1	17	NSCLC	PD		12
Nakeeb *et al*[57]	1	8	NSCLC	PD	3	
Total	13	6 (median)	NSCLC = 10 SCLC = 1	PD (7) DP (3) PPPD (3)	7 (median)	19 (median)

NSCLC: Non-small cell lung cancer; SCLC: Small cell lung cancer; NA: Not available; DP: Distal pancreatectomy; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; DOD: Dead of disease.

from RCC represent a favourable subgroup and surgical resection is recommended for these patients, whenever possible. However, a multidisciplinary approach has to be recommended and further studies are needed to establish the way to combine surgery with medical treatment in the different periods of the disease.

Considering the data available in the literature, it seems reasonable to consider surgery for pancreatic metastases from colorectal cancer a palliative treatment. However, an aggressive surgical approach may be considered in selected cases, in particular in symptomatic patients with isolated pancreatic metastasis.

Resection of melanoma metastatic to the pancreas appears to be only a palliative procedure. However, surgical resection may be considered in limited pancreatic disease with palliative intent. Even in the case of pancreatic metastases from breast cancer it is impossible to establish the course of the disease without surgical resection and to assess the real benefit in survival of the metastasectomy. However, in selected patients with a limited pancreatic disease, surgery may play a role in conjunction with chemotherapy, hormonal therapy and radiation therapy in the multimodality treatment of metastatic breast carcinoma. Solitary pancreatic metastases from lung cancer have a poor prognosis and treatment options for metastatic lung cancer lesions to the pancreas are mainly palliative. Finally, resection of pancreatic metastases from sarcoma is substantially justified in individual basis.

CONCLUSION

Pancreatic metastases, although uncommon, are an increasing clinical entity. Surgical resection is often advocated when the lesion is single and for patients fit to perform a pancreatectomy. The usefulness of pancreatic resection is mainly linked to the biology of the primary tumor metastasizing to the pancreas. The benefit of

metastasectomy in terms of patient survival has been observed for metastases from RCC, while for other tumors the role of surgery is mainly palliative. In fact, from our data and from a review of the literature, pancreatic surgery for metastases from colorectal cancer and melanoma may be considered for palliation, even if in selected cases surgical resection can be advocated in the multimodality treatment of metastatic colorectal cancer. Even in the case of pancreatic metastases from breast cancer, an aggressive surgical approach appears useful for good palliation in selected patients with a limited pancreatic disease. Patients with solitary metastases from lung cancer have a poor outcome and do not benefit from surgical resection. Finally, resection of pancreatic metastases from sarcoma is substantially justified only in very selected patients.

Patients with pancreatic metastases should be evaluated with a multidisciplinary approach, being surgery part of the multimodality treatment of these clinical entities. Further studies are needed to establish the way to combine surgery with medical treatments in the different metastatic diseases to the pancreas.

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2014 ADVANCES IN PANCREATIC CANCER

Pancreatic neuroendocrine tumor accompanied with multiple liver metastases

Tomohide Hori, Kyoichi Takaori, Shinji Uemoto

Tomohide Hori, Kyoichi Takaori, Shinji Uemoto, Department of Hepato-Pancreato-Biliary and Transplant Surgery, Kyoto University Hospital, Kyoto 606-8507, Japan
Author contributions: Hori T wrote the paper; Takaori K and Uemoto S supervised this paper.
Correspondence to: Tomohide Hori, MD, PhD, Department of Hepato-Pancreato-Biliary and Transplant Surgery, Kyoto University Hospital, 54 Shogoinkawara-cho, Sakyo-ku, Kyoto 606-8507, Japan. horit@kuhp.kyoto-u.ac.jp
Telephone: +81-75-7513651   Fax: +81-75-7513106
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Abstract

Pancreatic neuroendocrine tumor (P-NET) is rare and slow-growing. Current classifications predict its prognosis and postoperative recurrence. Curative resection is ideal, although often difficult, because over 80% of patients have unresectable multiple liver metastases and extrahepatic metastasis. Aggressive surgery for liver metastases is important to improve survival. Aggressive or cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve symptoms and prognosis. Liver transplantation was originally conceived as an ideal therapy for unresectable liver metastases. Unfortunately, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with unresectable liver metastases.

Key words: Gastroenteropancreatic neuroendocrine tumor; Pancreas; Liver metastasis; Liver surgery; Liver transplantation

Core tip: Pancreatic neuroendocrine tumor is rare. Current classifications predict its prognosis and postopera-

tive recurrence. Curative resection is often difficult, because over 80% of patients have unresectable multiple liver metastases and extrahepatic metastasis. Aggressive or cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve symptoms and prognosis. Liver transplantation was originally conceived as an ideal therapy for unresectable liver metastases. However, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases.

INTRODUCTION

Pancreatic neuroendocrine tumor (P-NET) is a rare and slow-growing tumor[1]. The American Joint Committee on Cancer stated a new TNM classification in 2009, based on tumor size, including direct invasion and lymphoid and distant metastases[2]. In 2010, the World Health Organization categorized gastroenteropancreatic neuroendocrine tumor (GEP-NET) into three categories (G1, G2 and G3) based on histopathological differentiation, proliferation index (Ki-67), neuroendocrine biomarkers (such as chromogranin A and synaptophysin), hormonal behavior, tumor size, direct invasion, and distant metastasis[3]. These classifications are useful for predicting the prognosis and postoperative recurrence[1]. Curative resection is ideal for this slow-growing tumor[1,4-6], and postoperative surveillance of at least 10 years is required, because long-term recurrence can occur after surgery[1].

Curative surgery is often difficult, because over 80% of P-NET patients already have unresectable multiple liver metastases and extrahepatic metastasis[1]. Some cur-

rent opinions suggest an expanded surgical indication for P-NET patients with liver metastases, because survival is improved[1,6-9]. Aggressive surgery for liver metastases or cytoreductive surgery for over 90% of the visible tumors are important to improve survival[6,9]. Cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve clinical symptoms and prognosis[1,6,9]. Liver transplantation (LT) was originally conceived as an ideal therapy for unresectable liver metastases[1,10].

Unfortunately, there is no clear consensus on the role and timing of surgery for primary tumor and liver metastases, although current reports refer to liver surgery including LT for unresectable liver metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with unresectable liver metastases. Here, we reviewed previous studies about therapeutic strategies for P-NET, with our regretful case.

RESECTION OF PRIMARY TUMOR

Approximately half of P-NETs are nonfunctioning[11], and tumors < 10-30 mm are not indications for surgery[1,6]. Functional P-NET should be removed even if the tumor is < 10 mm[1,6], because functional P-NET has malignant potential despite a small tumor size[1]. Some factors, such as young age, hormonal function, and surgical resection, are important for overall survival[6,12]. Seventy to ninety percent of enlarging P-NETs have malignant potential[1], and the aim of surgery for primary nonfunctioning tumor is to avoid malignant change and subsequent distant metastasis[6]. Although endoscopic ultrasonography with fine-needle aspiration biopsy is useful for determining the malignant potential and predicting prognosis[13-15], there are no definitive criteria regarding whether P-NET should be removed or observed based on tumor size[1,6]. Curative resection is considered as standard therapy in well-differentiated GEP-NET G1/G2 with a Ki-67 index of < 10%[1,4]. Cytoreductive surgery for primary tumor is indicated to reduce hormone levels and improve clinical symptoms[1,6,16], although the effects on prognosis are still controversial[1,5]. Overall, surgery for primary tumor should be curative resection[1,4-6], although palliative therapy may be indicated if there is a possibility of improvement of clinical symptoms, such as endocrine symptoms, oppression on surrounding organs by primary tumor, jaundice and oral passage disturbance[6,17].

RESECTION OF LIVER METASTASES

Curative surgery is often difficult, because over 80% of P-NET patients already have unresectable multiple liver metastases and extrahepatic metastasis[1]. Current opinions suggest extended surgical indications for P-NET patients with liver metastases, because survival is improved and P-NET is a slow-growing tumor[1,6-9]. For liver metastasis without extrahepatic metastasis, standard/aggressive surgery is the first choice for well-differentiated P-NET categorized as GEP-NET G1/G2[1,7,8]. Aggressive surgery for liver metastases and cytoreductive surgery for > 90% of the visible tumors are important to improve survival[6,9]. Cytoreductive surgery for liver metastases is indicated to reduce hormone levels and improve clinical symptoms and prognosis[1,6,9].

LT FOR UNRESECTABLE LIVER METASTASES

LT was originally conceived as an ideal therapy for advanced hepatic malignancy, because it eliminates the liver tumors and the potential for recurrence in the liver remnant[1,10]. LT for unresectable metastases has essentially been abandoned[10]. Several attempts to implement this strategy between 1960 and the 1980s showed poor results, although LT for early hepatocellular carcinoma has been established[18]. It is well known that highly selected P-NET patients with liver metastases may be candidates for LT[10,19-21]. The only prospective study recommended strict selection criteria for LT with curative intent (*i.e.*, low grade, removal of primary tumor, liver involvement < 50%, age < 55 years, and stable disease for ≥ 6 mo before LT)[21], and a study reported 96% overall survival and 80% disease-free survival[22]. However, it was also reported that P-NET patients with liver metastases who received LT had a follow-up term of no longer than 5.8 years, and the longest tumor-free survival was 5.1 years[23], and a high rate of tumor recurrence was reported at almost 60%[20].

Use of LT for extended indications always presents an ethical dilemma[10]. The United Network for Organ Sharing has generally held that LT for malignancy should be considered only when results are essentially equivalent to results with standard indications, generally requiring a 5-year survival rate of 60%-70%[10]. LT in selected GEP-NET patients has shown a 5-year recurrence-rate as low as 30%[21]. Previous results that indicate LT for P-NET[20-22] must be interpreted cautiously[10], especially given the global scarcity of liver grafts available[10]. These results should not justify LT at this time[10]. The Milan Criteria is maybe a better definition of selection criteria for LT[21]. In the last decade, selection criteria based on clinical presentation have been integrated with a proper histopathologic classification and diagnostic techniques[21]. In particular, Ki67 expression has been considered as a prognostic factor of risk of recurrence[21,24-28]. A Ki67 proliferation index of < 10% is a characteristic of well-differentiated tumor, which we have adopted as a cut-off value to consider GEP-NET patients for LT candidates[21,24]. Current studies suggest a growing consensus concerning LT for liver metastases of P-NET as follows[20,24-28]: (1) liver metastases of symptomatic or asymptomatic P-NET are unresectable; (2) disease is confined to the liver, and extrahepatic metastases are ruled out; (3) LT is indicated for well-differentiated P-NET categorized as GEP-NET G1/G2. Poorly differentiated P-NET categorized as GEP-NET G3 is considered as a contraindication for LT. Ki67 index < 10% is recommended; and (4) LT should not be associated with major extrahepatic

resection. Primary tumor should be removed before LT.

As described above, primary tumor should be removed before LT. However, optimal timing for LT in patients with stable versus progressive disease remains unclear[20]. In previous report, 83% of patients had undergone surgical treatment for primary tumor, and a 5-year overall survival has increased to 59% in relation with fewer patients presenting poor prognostic factors[20]. Favorable outcomes in cases of unknown primary tumor might suggest that a failure to detect the primary tumor before LT should not be considered as an absolute contraindication[20].

MANAGEMENT OF UNRESECTABLE LIVER METASTASES

For metastatic poorly-differentiated P-NET categorized as GEP-NET G3, cisplatin-based combination therapy is considered as the first-line therapy. Radiofrequency ablation, transarterial chemoembolization (TACE), transcatheter arterial infusion (TAI) and selective inhibitor of mammalian target of rapamycin are available as optional treatments[1]. Systemic biotherapy, such as somatostatin analog and interferon-α, is indicated for functional P-NET and postoperative recurrence[1].

Peptide receptor radionuclide therapy (PRRT) with radiolabeled somatostatin analogs is a novel treatment in patients with somatostatin receptor-expressing, well-differentiated and metastatic neuroendocrine tumors[29-31], and the PRRT with yttrium and/or lutetium is a potent therapeutic approach. On the other hand, transarterial radioembolisation [*i.e.*, selective internal radiotherapy (SIRT)] is an innovative therapy in liver-limited unresectable, neuroendocrine liver metastases[32-34]. SIRT is an effective treatment option for patients with metastatic liver disease in a salvage setting with acceptable toxicity.

OUR REGRETFUL CASE

A 39-year-old man was diagnosed with nonfunctioning P-NET in the pancreatic head, with multiple liver metastases. The tumor was 2.5 cm in diameter, and was histopathologically well-differentiated with a Ki-67 expression of < 10%. He was asymptomatic. Small but multiple metastases were detected in the liver, and no extrahepatic metastases were observed. We initially intended to control the liver metastases before resection of the primary tumor. To begin with, TACE/TAI were repeated. Thereafter, TACE/TAI, systemic chemotherapies and biotherapies were repeated. Although liver metastases seemed to be stable for a while, the primary tumor was enlarged even after therapy. At 3.5 years after initial diagnosis, the primary tumor became symptomatic. Liver metastases enlarged and massive swelling of the para-aortic lymph nodes was observed. Thereafter, palliative therapy was the main course of action. He died at 4.3 years after initial diagnosis. We understand that P-NET patients often have unresectable liver metastases at initial diagnosis[1],

and that surgical indications for P-NET with liver metastases should be determined individually in each case[6]. Resection of the primary tumor in metastatic nonfunctioning P-NET patients with unresectable liver metastases does not significantly improve survival[4]. Presence of liver metastases is a major prognostic factor for P-NET patients[1,20], and surgical management of liver metastases remains controversial[9]. In our case, we initially intended to control the liver metastases before resection of the primary tumor, because we considered liver metastases as the most important prognostic factor. Our decision at that time may have been consistent with previous opinions[1,4,6,9,20]. However, in our case, aggressive surgery for liver metastases seemed to be difficult even during a period of stable liver metastases, and resection of primary tumor is required before LT. We retrospectively regret that aggressive surgery for primary tumor and subsequent LT for unresectable liver metastases may have provided a better course in our case.

DISCUSSION

Currently, classification of GEP-NET is useful for evaluating malignancy, predicting prognosis, and determining therapeutic strategies[1,2]. Though this report focused surgical options for P-NET with liver metastases, novel managements (*i.e.*, PRRT and SIRT) are currently available for unresectable liver metastases, with acceptable side effects[29-34]. Effective and beneficial treatment options for P-NET patients with liver metastases should be carefully considered. From the viewpoint of surgical option, surgical indications for primary tumor[1,4-6,16] and hepatic surgery, including LT for liver metastases[1,10,20,24-28] have already been stated. However, it seems to be not easy to decide optimal timing of surgery for primary tumor and liver metastases. Currently, surgical procedures and devices are well developed, and the question is whether pancreatoduodenectomy or distal pancreatectomy is risky. We believe that pancreatic surgery is safe and beneficial for patients, if indicated.

In LT for P-NET patients, previous excellent reports focused on a prognostic factors for overall survival, a post-transplant risk of recurrence, a better selection criteria, a difference between P-NET and others, and an importance of the post-transplant surveillance[21,24,28]. There is a difference in behaviors between P-NET and the other tumors, the indication for LT for unresectable liver metastases is unique for P-NET[21,24]. Also, an importance of careful surveillance after LT due to the risk of recurrence was documented[21,24]. Tumor re-staging should be scheduled at least 4 times per year for the first two years and continued thereafter with progressively longer follow-up intervals[21].

Though we understand that any decisions cannot be made based on a single patient experience, we retrospectively speculate that a negative approach to aggressive surgery for primary tumor may have resulted in poor quality of life and deprived patient of the opportunity of LT for unresectable liver metastases. P-NET patient with

liver metastases could have been a candidate for initial surgery for primary tumor and might have had a chance of subsequent LT for unresectable metastases. Surgeons still face questions in deciding the best surgical scenario in patients with P-NET with liver metastases.

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2014 ADVANCES IN PANCREATITIS

Acute pancreatitis: The stress factor

Marcelo G Binker, Laura I Cosen-Binker

Marcelo G Binker, Laura I Cosen-Binker, CBRHC Research Center, 1053 Ciudad Autónoma de Buenos Aires, Buenos Aires 1426, Argentina

Laura I Cosen-Binker, Department of Molecular and Cell Biology, Henry M Goldman School of Dental Medicine, Boston University, Boston, MA 02118, United States

Author contributions: Binker MG and Cosen-Binker LI contributed equally to this work.

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Correspondence to: Dr. Laura I Cosen-Binker, Department of Molecular and Cell Biology, Henry M Goldman School of Dental Medicine, Boston University, 72 East Concord Street - Evans Building - Room 408, Boston, MA 02118, United States. licb@bu.edu

Telephone: +1-617-4141084 Fax: +1-617-4141041

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Abstract

Acute pancreatitis is an inflammatory disorder of the pancreas that may cause life-threatening complications. Etiologies of pancreatitis vary, with gallstones accounting for the majority of all cases, followed by alcohol. Other causes of pancreatitis include trauma, ischemia, mechanical obstruction, infections, autoimmune, hereditary, and drugs. The main events occurring in the pancreatic acinar cell that initiate and propagate acute pancreatitis include inhibition of secretion, intracellular activation of proteases, and generation of inflammatory mediators. Small cytokines known as chemokines are released from damaged pancreatic cells and attract inflammatory cells, whose systemic action ultimately determined the severity of the disease. Indeed, severe forms of pancreatitis may result in systemic inflammatory response syndrome and multiorgan dysfunction syndrome, characterized by a progressive physiologic failure of several interdependent organ systems. Stress occurs when homeostasis is threatened, and stressors can include physi-cal or mental forces, or combinations of both. Depending on the timing and duration, stress can result in beneficial or harmful consequences. While it is well established that a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis, the worsening effects of chronic stress on the exocrine pancreas have received relatively little attention. This review will focus on the influence of both prior acute-short-term and chronic stress in acute pancreatitis.

Core tip: Depending on the timing and duration, stress can result in beneficial or harmful consequences. Regarding the exocrine pancreas, a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis. This protection is conferred by distinct heat shock proteins (HSP) including HSP27, HSP60 and HSP70. Conversely, chronic stress increases the susceptibility of the exocrine pancreas, aggravating pancreatitis episodes. These worsening effects are mainly mediated by tumor necrosis factor alpha.

INTRODUCTION

Acute pancreatitis is an inflammatory disorder of the pancreas with an overall mortality of approximately 5%[1]. Etiologies of pancreatitis vary, with gallstones accounting for the majority of all cases, followed by alcohol. Other causes of pancreatitis include trauma, isch-

emia, mechanical obstruction, infections, autoimmune, hereditary, and drugs[2].

The main events occurring in the pancreatic acinar cell that initiate and propagate acute pancreatitis include inhibition of secretion, intracellular activation of proteases, and generation of inflammatory mediators[3]. These cellular events can be correlated with the acinar morphological changes (retention of enzyme content, formation of large vacuoles containing both digestive enzymes and lysosomal hydrolases, and necrosis), which are observed in the well-established *in vivo* experimental model of supraphysiological cerulein-induced pancreatitis[4], as well as in human acute pancreatitis[5]. Chemokines released from damaged pancreatic cells attract inflammatory cells, whose systemic action ultimately determined the severity of the disease. Indeed, severe forms of pancreatitis may result in systemic inflammatory response syndrome and multiorgan dysfunction syndrome, characterized by a progressive physiologic failure of several interdependent organ systems[6].

Stress can be defined as "threatened homeostasis", and stressors can include physical or mental forces, or combinations of both. The reaction of an individual to a given stressor involves the stimulation of pathways within the brain leading to activation of the hypothalamic-pituitary-adrenal axis and the central sympathetic outflow[7]. This can result in visceral hypersensitivity through the release of different substances, such as substance P and calcitonin gene-related peptide from afferent nerve fibers[8].

The main source of pancreatic innervation comes from both vagus nerves and the celiac ganglion complex. The cephalic segment is innervated by the right celiac complex and the hepatic and mesenteric plexus coming from the right vagus. The splenic segment is innervated by the left celiac nerve and the splanchnic nervous network. Except for the gastro-duodenal branches network, most of the nerves enter the gland by its periphery and concentrate in the cephalic segment, which exhibits an important number of ganglion cells. These characteristics of the macroscopic innervation decrease in a significant and progressive fashion towards the splenic segment[9,10].

While it is well established that a previous acute-short-term stress decreases the severity of experimentally-induced pancreatitis[11-17], the worsening effects of chronic stress on the exocrine pancreas have received relatively little attention[18-20]. This review will focus on the influence of both prior-acute-short-term and chronic stress in acute pancreatitis.

ACUTE STRESS

Preceding acute-short-term stress is a well-known inductor of cellular protection against numerous pathological conditions, including renal ischaemia, heart ischaemia, brain ischaemia, enterocolitis and pancreatitis[11-17,21-25]. Exposure of organisms to an initial sublethal stress leads

to the synthesis of heat shock proteins (HSP) and confers protection against further stress[26]. HSP comprise a highly conserved family of proteins with molecular sizes ranging from 10 to 110 kDa. These molecular chaperones are involved in synthesis, folding, transport and degradation of proteins, and can be induced by stressful conditions such as infection, inflammation, hypoxia, starvation, heat shock, water immersion, and oxidative stress[27-29].

The eventual protection conferred by acute stress-induced HSP in pancreatitis, seems to be stressor- and disease-inducer-dependent[30,31]. Water immersion and heat shock induce pancreatic HSP60 and HSP70, respectively, and protect rats from cerulein-induced acute pancreatitis by inhibiting autophagy, which prevents the subcellular redistribution of cathepsin B and the activation of trypsinogen[14,32,33]. Additionally, hyperthermia- or chemical-stimulated HSP70 also decrease the production of inflammatory mediators by downregulation of NF-κB[34,35]. Remarkably, transgenic mice knock-out for HSP70 ($HSP70.1^{-/-}$) develop spontaneous activation of pancreatic trypsinogen[36]. However, transgenic knock-in mice over-expressing HSP72 do not exhibit protection for development of cerulein-induced acute pancreatitis, but HSP72 over-expression accelerates tissue injury recovery by lessening NF-κB signaling[37]. Heat shock also induces pancreatic protection against cerulein hyperstimulation by upregulating HSP27[38]. Indeed, over-expression of HSP27 preserves the actin cytoskeleton of pancreatic acinar cells and protect against cerulein-induced pancreatitis in a specific phosphorylation-dependent manner[39]. HSP27 exerts a similar protective effect in coronary arteries[40]. Vessels (endothelial and/or smooth muscle cells) from patients with ischemic heart disease exhibit decreased levels of HSP27 (in particular phospho-HSP27), which correlates with destabilization of the actin cytoskeleton[40]. Regardless of the underlying mechanism, disorganization of the actin cytoskeleton is associated with dysregulation of pancreatic enzyme secretion[41]. Interestingly, HSP27 seems to coordinate activity with other HSP members to provide the full extent of resistance to injury[42]. For instance, , depletion of HSP70 in renal cells does not impede association of HSP27 with actin, but prevents maximal cytoprotective effect against energy depletion[42].

Other pancreatitis-induced models exhibit some differences with the previously mentioned, secretagogue hyperstimulation. Thus, hyperthermia protects against subsequent L-arginine-induced acute pancreatitis in rats by increasing pancreatic expression of HSP70 and HPS27, and phosphorylation of HSP27, but without changing HSP60 levels[15,43]. As observed in the cerulein model, transgenic mice over-expressing HSP72 do not exhibit protection for L-arginine-induced acute pancreatitis[37]. However, HSP72 over-expression does not accelerate tissue injury recovery in L-arginine treated animals[37]. Although both hot and cold water immersion induce pancreatic HSP72 and HSP60, respectively, only cold water
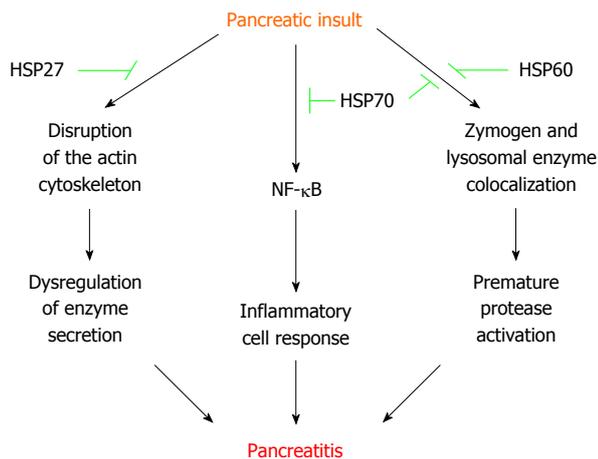
Figure 1 Hypothetical mechanisms underlying prior-acute-short-stress protects against pancreatitis. Pancreatic insults may provoke dysregulation of enzyme secretion, premature protease activation and inflammatory acinar response, which result in the development of pancreatitis. Different stressors such as hyperthermia, hypothermia, hypoxia, energy depletion and chemicals, can induce pancreatic heat shock proteins (HSP) by a prior-acute-short-stress exposition. Distinct HSP avoid the disruption of the actin cytoskeleton, zymogen/lysosomal enzyme colocalization and activation of the pro-inflammatory nuclear factor-kappa beta (NF-κB) caused by the pancreatic insult. These HSP-mediated effects seem responsible for the protection against pancreatitis. The specific pathway inhibited by each HSP is depicted in green.

immersion slightly protect rats from sodium tauracholate-induced acute pancreatitis, pointing the transcendence of the subcellular redistribution of cathepsin B in this necrohemorrhagic pancreatitis model[13].

Nevertheless, prior-acute-short-term stress protects against pancreatitis by distinct HSP, which seem to exert its beneficial effects through different pathways (Figure 1).

CHRONIC STRESS

Chronic stress has been proved to increase the susceptibility of different rat organs, such as the small intestine, colon and brain, to inflammatory diseases[8,20,44-46], as well as to aggravate atherosclerotic lesions in mice[47].

Even though oxidative stress and inflammation each occur in the pancreas during the early stage of supra-maximal cerulein-induced acute pancreatitis model, neither oxidative stress nor an inflammatory insult alone cause the characteristic changes of acute pancreatitis[48]. However, chronic stress leaves the exocrine pancreas susceptible to pancreatitis by submaximal cerulein stimulation[20]. Pancreatic tissue from rats chronically exposed to restraint exhibit measurable levels of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) as well as a low but detectable leukocyte infiltrate and myeloperoxidase activity[20], suggesting leukocytes as a feasible source of TNF-α induced by chronic stress. Interestingly, *in vitro* incubation of mice pancreatic acini with phorbol-12-myristate-13-acetate-activated neutrophils or macrophages directly induce intracellular trypsinogen activation and cell death, being protease

activation and necrosis mediated by leukocyte-secreted TNF-α in a cathepsin-B and calcium-dependent manner[49].

TNF-α has an important role in various biological functions, including cell proliferation, cell differentiation, survival, apoptosis and necrosis[50], and in stress-related inflammatory disorders[45-47,51]. For a long time, it has been known that TNF-α participates in the inflammatory cascade which propagates pancreatitis[52]. Nevertheless, its relevance in the genesis of this debilitating disease only recently captured the attention of research investigation[20,49].

Secretion of TNF-α by several stress stimuli has been demonstrated *in vitro* in many cell types, including pancreatic acinar cells[53-60], and *in vivo* in different tissues[47,51,61-63]. Our lab has shown that *in vitro* hypoxia-reoxygenation conditions also induce TNF-α secretion by acinar cells[20]. These conditions are concomitant with ischemia-reperfusion processes, which can be the result of microcirculatory disturbances generated by stress[64]. Indeed, local pancreatic blood flow is reduced by stress[65]. Hence, alternate vasoconstriction and vasodilatation leading to tissue ischemia and reperfusion could reflect another putative local origin of chronic stress-derived TNF-α found in the pancreatic tissue. This is supported by the increased levels of the transcription factor hypoxia inducible factor 1 alpha (HIF-1α) observed in experimentally stressed rats[20]. HIF-1α is induced by hypoxic conditions and is involved in different inflammatory processes, such as dermatitis, rheumatoid arthritis[66], and also pancreatitis[67].

Different reports evaluated the response of pancreatic acinar cells to exogenous TNF-α, showing disruption of the typical filamentous actin distribution[20,68]. A similar redistribution of actin from apical to basolateral membranes was observed in pancreatic acini supra-stimulated with CCK[69]. While TNF-α alone does not stimulate amylase secretion in human pancreas[70] or in isolated rat pancreatic acini[20,68], it certainly inhibits sub-maximal CCK-stimulated amylase secretion[20]. Although necessary, the inhibition of pancreatic enzyme secretion alone is not sufficient to induce pancreatitis[3]. Nonetheless, TNF-α also activates pancreatic acinar nuclear factor-κB (NF-κB), a key transcriptional regulator of the expression of inflammatory molecules[20,68,71,72]. Consistently, rat pancreatic acinar cells treated with high doses of exogenous TNF-α, exhibit a notable increase in the production of cytokines interleukin (IL)-1β, IL-4, IL-6, IL-10, as well as TNF-α[73].

TNF-α has been shown to regulate the activity of distinct protein kinase C (PKC) isoforms in diverse cell types, including the pancreatic acinar cell[72,74,75]. PKC family comprises at least 12 members differing in tissue distribution and activation requirements. There are three subclasses: classical PKC isozymes (-α, -β1, -β2, and -γ), which require calcium and are activated by diacylglycerol and phorbol ester; the novel PKC isozymes (-δ, -ε, -η, and -θ), which are activated by diacylglycerol and phor-
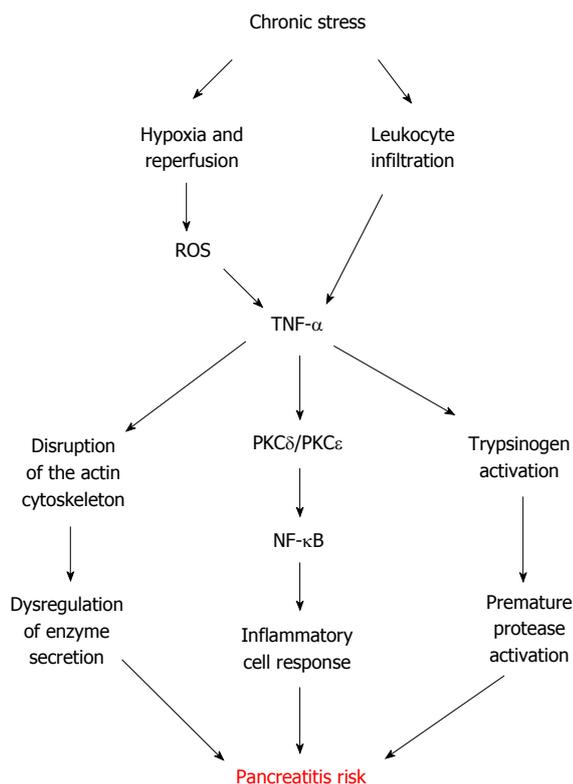
Figure 2 Hypothetical mechanisms involved in chronic stress sensitizes to pancreatitis. Chronic stress compromises the exocrine pancreas by generating ischaemia and reperfusion processes, as well as attracting leukocytes to the pancreatic parenchyma. Ischaemia and reperfusion induce hypoxia and reoxygenation conditions that generate the intrapancreatic reactive oxygen species (ROS) responsible for acinar tumor necrosis factor-alpha (TNF-α) production. TNF-α released from both pancreatic acinar cells and leukocyte infiltrate, impact on pancreatic acinar cells producing disruption of the actin cytoskeleton (redistribution from apical to basolateral membrane), a protein kinase C delta (PKCδ)- and PKC epsilon (PKCϵ)-mediated activation of the transcription factor nuclear factor-kappa beta (NF-κB), and an increase in levels of active trypsin. Dysregulation of enzyme secretion, induction of inflammatory acinar response and premature intra-acinar protease activation associated to these pathological pathways sensitize the exocrine pancreas to pancreatic insults and increase the risk to develop pancreatitis.

bol ester independently of calcium; and the atypical PKC isozymes (-λ, -ι, and -ζ), which are calcium independent and not responsive to phorbol ester. Rat pancreatic acini express the α, δ, ϵ, and ζ PKC isozymes[76]. Changes in PKC activity are associated with inflammation in a variety of tissues, including skin, kidney, intestine, and pancreas[77-80]. Specifically, PKC-δ and PKC-ϵ regulate the signal transduction pathways implicated in the pathophysiological activation of NF-κB and trypsinogen in rat pancreatic acini[72,81]. TNF-α activates both PKC-δ and PKC-ϵ in rat pancreatic acini[72], which convert physiological CCK concentrations into phytopathogenic concentrations[20]. Different studies have consistently shown that modulation of PKC activity sensitizes acinar cells to physiological secretagogue treatments, resulting in harmful levels of NF-κB and trypsin activity[81,82]. In agreement, TNF-α plus submaximal CCK pathologically activates NF-κB and trypsinogen in rat pancreatic acini, and induced both apoptosis and necrosis[20]. However,

pancreatic acini response from rats seems to differ from that observed in mice, since TNF-α by itself only induces trypsinogen activation and necrosis in mice, with an extent comparable to supramaximal cerulein stimulation[20,49]. This could be a concentration-dependent effect or relative to differences between species, which is well-documented for experimentally-induced pancreatitis in rodents[83-86], but further studies are required to address this disparity in pancreatic acinar response to exogenous TNF-α.

Summarizing this topic, chronic stress appears as a risk factor to develop pancreatitis by sensitizing the exocrine pancreas through TNF-α, which seems to exert its detrimental effects through different pathways (Figure 2).

CONCLUSION

Depending on the timing and duration, stress can result in beneficial or harmful consequences for the exocrine pancreas. Prior acute-short-term stress could be useful for high-risk procedures such as endoscopic retrograde cholangiopancreatography. Conversely, the management of chronic stress appears critical for patients with risk of pancreatitis. Nonetheless, the mechanisms underlying protection by previous-acute-short-term stress as well as burden by chronic stress, have to be further explored.

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2014 ADVANCES IN PANCREATITIS

Surgical and interventional management of complications caused by acute pancreatitis

Feza Y Karakayali

Feza Y Karakayali, Baskent University, Faculty of Medicine, Department of General Surgery, Ankara 06490, Turkey
Author contributions: Karakayali FY contributed to the study idea, study design, literature search, manuscript writing and final revision of the article.
Correspondence to: Feza Y Karakayali, MD, Associate Professor of Surgery, Baskent University, Faculty of Medicine, Department of General Surgery, Fevzi Cakmak Cad. 5. Sok. No. 48 Bahcelievler, Ankara 06490, Turkey. fezaykar@yahoo.com
Telephone: +90-532-6455407 Fax: +90-532-6455312
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Abstract

Acute pancreatitis is one of the most common gastrointestinal disorders worldwide. It requires acute hospitalization, with a reported annual incidence of 13 to 45 cases per 100000 persons. In severe cases there is persistent organ failure and a mortality rate of 15% to 30%, whereas mortality of mild pancreatitis is only 0% to 1%. Treatment principles of necrotizing pancreatitis and the role of surgery are still controversial. Despite surgery being effective for infected pancreatic necrosis, it carries the risk of long-term endocrine and exocrine deficiency and a morbidity and mortality rate of between 10% to 40%. Considering high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being explored by gastrointestinal surgeons, radiologists, and gastroenterologists. Since 1999, several other minimally invasive surgical, endoscopic, and radiologic approaches to drain and debride pancreatic necrosis have been described. In patients who do not improve after technically adequate drainage, necrosectomy should be performed. When minimal invasive management is unsuccessful or necrosis has spread to locations not accessible by endoscopy, open abdominal surgery is recommended. Additionally, surgery is recognized as a major determinant of outcomes for acute pancreatitis, and there is general agreement that patients should undergo surgery in the late phase of the disease. It is important to consider multidisciplinary management, considering the clinical situation and the comorbidity of the patient, as well as the surgeons experience.

Core tip: The surgery and its timing are contentious regarding treatment of severe acute pancreatitis and related complications. Many studies showed that "early" open surgery has been accompanied often by higher mortality and morbidity rates, and should be the next step in treating severe acute pancreatitis complications, when minimally invasive management fails. In this review article, current treatment options and results are discussed.

INTRODUCTION

Acute pancreatitis (defined as the acute nonbacterial inflammatory condition of the pancreas) is derived from early activation of digestive enzymes inside acinar cells, with varying compromising of the gland itself, nearby tissues, and other organs. It is well known that several situations develop into acute pancreatitis, but the mechanisms and how those mechanisms develop the disease remain

unclear. Why do some individuals develop edematous pancreatitis and others develop a more severe necrotic pancreatitis? Knowledge regarding pancreatitis pathogenesis may have important implications in prevention and treatment of the disorder. If the early events that generate the inflammatory process are understood - and if pro- and anti-inflammatory factors that modulate the severity of the disease are known - treatment can be implemented so the process will not happen or possible associated complications will be minimized[1].

Acute pancreatitis is one of the most common gastrointestinal disorders requiring acute hospitalization worldwide, with a reported annual incidence of 13 to 45 cases per 100000 persons[2]. In the United States, it is the third most common gastrointestinal disorder requiring acute hospitalization[3]. In the United States alone, acute pancreatitis leads to 270000 hospital admissions annually and in-patient costs exceeding 2.5 billion dollars[4].

It is rare in childhood but may occur at any age (according to recent publications[5,6], median age, 55-58 yr). Acute biliary pancreatitis is more common in women, and alcoholic pancreatitis is more common in middle-aged men[6].

Although most patients with acute pancreatitis recover without sequelae, between 10% to 20% will have a more complicated clinical course with higher risks of morbidity and mortality[7]. Severe acute pancreatitis (SAP) requires prolonged hospitalization, frequently including a stay in the intensive care unit (ICU) because of organ dysfunction[8].

Severe pancreatitis is associated with a mortality of 15% to 30%, whereas mortality from mild pancreatitis is only 0% to 1%, and organ failure is the most important determinant of mortality in acute pancreatitis. However, in approximately 30% of patients with necrotizing pancreatitis, a secondary necrotic infection occurs, mostly 3 to 4 wk after the onset of necrotizing pancreatitis[9]. If left untreated, mortality of infected necrosis approaches 100%[3,10]. Initial treatment of SAP is primarily medical, and these patients require intensive organ support[11,12]. Surgery for SAP is a morbid procedure associated with complications in 34% to 95% of patients, and mortality in 11% to 39%[13,14]. Surgery may lead to long-term pancreatic insufficiency[14,15]. The high mortality rate encountered with surgery reflects the hazards of operating on critically ill septic patients, often with multiorgan failure[16].

Surgery and its timing are the focus of contention when treating SAP. Decades ago, some experts used laparotomy in the early phase of SAP to debride and drain the retroperitoneal infected necrosis[17,18]. However, studies have shown that "early" surgery is often accompanied by higher mortality[19,20], and several studies also have shown that there is success with some SAP patients with retroperitoneal infected necrosis, conservatively managed without high-risk surgical intervention; therefore, many experts advocated delayed surgery[20,21]. In recent decades, higher mortality rates during early surgery resulted from those SAP cases that underwent traditional laparotomy

(which may cause severe trauma) to debride and drain the retroperitoneal infected necrosis[22]. After several studies showed that high mortality rates for severe necrotizing pancreatitis came with early surgery, the 2002 International Acute Pancreatitis guidelines recommended avoiding surgical intervention during the first 14 d after onset, unless there was progressive multiple organ failure and clinical deterioration. Subsequent studies have suggested that morbidity and mortality rates can be reduced further if surgery is delayed beyond 28 to 30 d[9], because the extended interval allows sufficient demarcation between normal and necrotic tissue, reducing the risk of inciting overwhelming postoperative septic and systemic inflammatory responses, and the risk of intraoperative injury to surrounding organs and hemorrhage[23].

Faced with high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being increasingly explored by gastrointestinal surgeons, radiologists, and gastroenterologists[24]. As technical ability and endoscopic tools have gradually become more precise, the mortality rates of patients with severe pancreatitis have improved, and there are fewer complications compared to those having open debridement treatment[25]. Percutaneous catheter drainage (PCD), endoscopic transgastric procedures, and a minimally invasive approaches all have been proposed as alternatives to open necrosectomy[16]. When minimal invasive management is unsuccessful or necrosis has spread to locations not accessible by endoscopy, open abdominal surgery is recommended[25].

CLASSIFICATION AND SCORING

The Atlanta Classification system for acute pancreatitis came about as a result of the Atlanta Symposium of 1992, and, despite there being some confusion over definitions, it has been a practical aid for health care providers[11]. Since then, with improvements in the understanding of organ failure and necrotizing pancreatitis, and in diagnostic tools, some revisions have been made through a working group consultation with eleven international pancreatic societies[26]. The fourth draft, in current use, contains a clinical assessment of severity and the previous confusing definitions concerning local complications have been further clarified. The criteria for the diagnosis of acute pancreatitis, the differences between the two forms (*i.e.*, interstitial edematous pancreatitis and necrotizing pancreatitis), the three categories of severity of acute pancreatitis (mildly acute, moderately severe acute, and severe acute)[27,28], and the morphology observed in diagnostic images of pancreatic and peripancreatic collections brought about by complications are now more clearly set out.

Criteria to help in classifying severity are the presence of transient organ failure (that which is present for less than 48 h), persistent organ failure (continuing for more than 48 h), and local (such as, peripancreatic fluid or acute necrotic collections) or systemic complications (such

as exacerbations of underlying comorbidities related to the acute pancreatitis)[29-31].

Scoring systems

Attempts to define objective criteria for assessing disease severity and prognosis were pioneered in the 1970s by Ranson et al[32] and Blamey et al[33]. The 2 scoring systems include basic laboratory data and clinical variables obtained 48 h after hospital admission. In subsequent years, these scoring systems have found widespread application and have undergone numerous modifications. Several large studies have shown a close correlation between advanced age and nonsurvival in acute pancreatitis[34-36]. Advanced age often is associated with comorbidities (e.g., cardiovascular disease, diabetes, and overall decreased biological resistance)[36], and therefore, increases risk of fatal outcome. Comorbidities have been included in multiple parameter scoring systems such as the Acute Physiology and Chronic Health Examination (APACHE) Ⅱ system, the most widely used index for early risk stratification[37]. Although more recent iterations of this scoring system have been developed, the advantages of the APACHE Ⅱ are its familiarity, its objective nature, and its ability to be calculated at any time during a patient's hospital stay. Use of the APACHE Ⅱ in clinical practice has several important limitations (e.g., the requirement for multiple parameters and an online calculator - versions of which are widely available on the Internet)[38]. As a result, several additional scoring systems have been developed for bedside application.

A more recent scoring system developed for use during the first 24 h of admission to the hospital is the Bedside Index of Severity in Acute Pancreatitis[7]. This system was derived using data from 17992 patients and validated on a population of 18256 patients in the United States. This 5-factor scoring system has a similar accuracy as the APACHE Ⅱ for predicting death in the initial retrospective study and in several subsequent prospective cohort studies[39]. The Bedside Index of Severity in Acute Pancreatitis is a simplified scoring system that can be applied easily in the earliest phases of acute pancreatitis helping identify those patients with an increased risk of death.

DEFINITION AND COMPLICATIONS

Defining the severity of acute pancreatitis

There are three good reasons for defining the severity of acute pancreatitis: the first being diagnosing those patients who may need aggressive early treatment in cases of severe acute form; the second is the identification of patients who may need to be transferred to a specialist care unit; and the third is that placing these patients into sub-groups according to particular complications will aid the specialists to whom they are transferred[26].

Mild acute pancreatitis

Patients without organ failure or complications are classified as having mild acute pancreatitis. They are usually discharged at an early stage, do not need pancreatic imaging, and death as a result of the disease is extremely uncommon[40].

Moderately severe acute pancreatitis

This is diagnosed when transient organ failure, local complications (such as prolonged abdominal pain, leukocytosis, or fever caused by peripancreatic collections, or if the patience can not feed normally), or systemic complications (such as when coronary artery disease or chronic lung disease is made worse as a result) are present. This form of the disease can resolve itself without treatment (when transient organ failure or acute fluid collection is involved) or specialist care may be needed (when extensive sterile necrosis is present, but organ failure is not). The chance of death as a result of this form is lower than in cases of the severe acute form[27].

Severe acute pancreatitis

This is diagnosed when there is persistent single or multiple organ failure, resulting from systemic inflammatory response syndrome caused by cytokine cascades at an early stage[30,31,41,42], which can complicate the pancreatitis, lead to other complications, and increase the risk of death (a 36% to 50% mortality rate), commonly due to infected necrosis, if this is in the first few days of the disease[31,41,42].

One systematic review into deaths caused by necrosis in the absence of organ failure (11% of patients) resulted in a four tier grading of severity being proposed[28], while two large Dutch studies came up with a figure of 6%[43,44]. The differences in morphological characteristics of local complications and their different treatments need to be determined to prevent mortality.

Necrotizing pancreatitis

Necrosis, which affects between 5% and 10% of patients, generally involves both the pancreas and peripancreatic tissue, although sometimes just the peripancreatic tissue, and, even more rarely, only the pancreatic parenchyma.

With patients who have peripancreatic necrosis, as in those with interstitial edematous pancreatitis, the pancreas enhances normally on contrast-enhanced computed tomography, but morbidity is increased and intervention rates are higher[40,45,46]. The progression of both pancreatic and peripancreatic necrosis varies, remaining solid or liquefying, becoming infected or remaining sterile, persisting for a long time or gradually disappearing.

Infected pancreatic necrosis

Both forms of necrosis can become infected, but the majority of evidence shows no certain correlation between its extent, the risk of infection, and its duration, although it is not common in the first week[9,43]. Its diagnosis is crucial as antibiotic and other necessary treatments need to be applied as soon as possible[47]. If computed tomography (CT) scans show up extraluminal gas in the pancreas or peripancreatic tissue, or if biopsies detect

bacteria and/or fungi on Gram stains and cultures, then infection is highly likely[48]. Signs of suppuration may also be evidence of liquefaction and will increase over time. Despite the first version of the Atlanta Classification defining a localized collection of purulent material without significant necrotic material as a pancreatic abscess, the term was found to be unhelpful and is not used in the revised version. Secondary infections have been found to increase the the chances of morbidity and death[49].

Acute pancreatitis complications

Defining organ failure: Organ failure in the respiratory, cardiovascular, and renal systems is defined using the modified Marshall scoring system: a score of two or more for one of these systems is sufficient[50]. This system is preferred over the Sepsis-related Organ Failure Assessment scoring system, used in critical care units, as it is easier to use and gives objective results, although both systems could be used to help stratify the severity of organ failure[51].

Defining local complications: The original Atlanta Classification was useful because it recognized the differences between uncomplicated interstitial pancreatitis and acute pancreatitis with local complications[11]. Local complications (such as acute peripancreatic fluid collection, pancreatic pseudocyst, acute necrotic collection, walled-off necrosis, gastric outlet dysfunction, splenic and portal vein thrombosis, and colonic necrosis) and their clinical consequences are now better understood and described. Signs that these problems may be present are persistent abdominal pain, secondary serum pancreatic enzyme activity increases, organ dysfunction getting worse, and symptoms of sepsis (*i.e.*, fever, white blood cell increases, *etc.*), although imaging may be necessary for correct diagnosis[26].

The definitions of pancreatic fluid collections are based on the revised Atlanta classification by the Acute Pancreatitis Classification Working Group and are described as follows: (1) Acute peripancreatic fluid collections (APFC): these are not connected to necrosis, occur in the first four weeks of acute pancreatitis, are entirely liquid, found in or near the pancreas, and have no fibrous wall or granulation tissue. Those which resolve themselves or have no symptoms need no treatment and are not classed as severe acute pancreatitis; (2) Pseudocyst: a collection of pancreatic juice, containing no solid necrotic material, enclosed by a wall of fibrous or granulation tissue resulting from acute pancreatitis, pancreatic trauma, or chronic pancreatitis. They are a result of the main pancreatic duct or its intrapancreatic branches being disrupted in the absence of pancreatic parenchymal necrosis and causing pancreatic juice to leak persistently and collect, usually after the first month; (3) Infected pseudocyst: this contains purulent liquid with no solid necrotic material (although there may be other solid debris) and can be diagnosed by following the patient's clinical course or through the presence of gas on CT scans; (4)

Post-necrotic pancreatic/peripancreatic fluid collections (PNPFC): fluid collections associated with necrotizing pancreatitis, containing fluid and necrotic tissue, which over the course of weeks, evolve into a necrotic fluid collection with liquid and solid debris; and (5) WON: these are formed because of encapsulation of the PNPFC over time in a thickened wall of fibrous or granulation tissue without an epithelial lining at the interface of necrotic tissue, generally maturing after the first month of necrotizing pancreatitis. Walled-off necrosis, resulting from necrotic pancreatic parenchyma and/or necrotic peripancreatic tissue, can be sterile or infected, and there can be many of them, sometimes in locations distant from the pancreas[52]. Walled-off necrosis helps to distinguish the necrotic tissue from the parenchyma, thereby lessening the chances of bleeding and the loss of vital tissue during surgery, although this can result in pancreatic exocrine and endocrine deficiency[53,54].

Bradley *et al*[55] suggested a conservative approach to sterile pancreatic necrosis, although the Acute Pancreatitis Classification Working Group found that patients may continue to be ill even when there was no infection[26,55]. Secondary infection, which usually occurs two to four weeks after primary infection, commonly results in sepsis, multi-organ failure, and patient mortality[56]. High Ranson's and APACHE-Ⅱ scores are good indicators of the possibility of death, and even those with severe sterile necrosis have a high mortality rate if their overall health is not good.

Defining systemic complications

Systemic complications are classed as those arising from already existent complaints, such as coronary artery disease, or chronic lung disease, made worse by the acute pancreatitis. The Acute Pancreatitis Classification Working Group made a distinction between these and persistent organ failure, the latter being the main feature of the severe acute form.

TREATMENT

Management of infected pancreatic necrosis

Pancreatic necrosis surgery, the principles of which were laid out by Moynihan in 1925[57], involves isolating the pancreas from the abdominal cavity and cellular fat spaces, and draining the amassed peripancreatic fluid. The aim is to check the sepsis and control the release of pro-inflammatory mediators. The combination of debriding the necrotic tissue and removing retro-peritoneal debris and exudate is carried out in order to preserve the organ. Four principle surgical methods are recommended: (1) being necrosectomy alongside open packing[58]; (2) being planned, staged relaparotomies with repeated lavage[21]; (3) being closed continuous lavage of the lesser sac and retro-peritoneum[59]; and (4) being closed packing[60].

These days, the third method is most often used to remove post-operative residual pancreatic necrosis as it has the lowest rate of morbidity[24,53]. Surgery has the pos-

sibility of saving the patient's life, but it carries a high risk of morbidity and mortality, between 4% and 10%, and possible long-term endocrine and exocrine deficiency[25]. In addition, timing of surgery has been increasingly recognized as a major determinant of outcome in acute pancreatitis, and there is general agreement that patients must undergo operation in the late phase of the disease. However, the definition of late differs between studies[53,61]. It has been reported that mortality from necrotizing pancreatitis can be reduced by avoiding surgical therapy or by postponing surgery until the late stage of the disease[62].

Despite the availability of several clinical (Ranson criteria, acute physiology and chronic health evaluation Ⅱ score, and APACHE Ⅱ) and radiologic grading systems (Balthazar scoring system, modified computerized tomography severity index), there is no consensus on accurately predicting the best treatment strategy and outcome after acute necrotizing pancreatitis[63-65].

The treatment principles of necrotizing pancreatitis and the role of surgery remain controversial. In the 1990s, more than 60% of patients with the disease were treated surgically[18]. In 1991, Bradley and Allen defined pancreatic necrosis as the principal determinant of survival in acute pancreatitis, but they recommended conservative treatment of sterile necrosis in selected cases[55]. Guidelines of the International Acute Pancreatitis recommend doing a fine-needle aspiration biopsy in patients with necrotizing pancreatitis and signs of sepsis. Once fine-needle aspiration biopsy-proven infection of necrosis has been shown, it is considered an indication for surgery[53].

Recent reports have shown that a subset of patients with SAP developing infected fluid collection, pancreatic necrosis, or pancreatic abscess can be managed by PCD[66]. It was hypothesized that simple drainage with regular-bore (12- to 14-Fr) percutaneous catheters is an effective therapeutic option. This recommendation is based on the premise that is not necessary to remove all necrotic tissue to successfully treat patients with infected pancreatic necrosis. By performing drainage of infected fluid under pressure, the clinical condition might improve and the necrotic tissue may successfully be dealt with by the patient's immune system. The goal of drainage has been to remove infected fluid rather than the necrosis[67]. However, PCD used for infected pancreatic necrosis has been criticized for its poor ability to remove solid debris.

Percutaneous drainage is usually performed under computed tomography, whereas sonographically controlled PCD rarely has been reported[68]. The success rate of percutaneous catheter drainage in infected pancreatic necrosis varies and ranges from 0% to 78%[43,69]. van Baal et al[70] reported a meta-analysis, which included 384 patients from 11 studies, of PCD as a primary treatment for necrotizing pancreatitis. Surgical necrosectomy could be avoided in 56% of the patients and the overall mortality rate was 17%. However, infected necrosis was confirmed in only 71% of the patients.

In a recent report, authors aimed to identify factors that led to surgical intervention after initial management with PCD, and also to identify a subgroup of patients where PCD alone would be effective. Twenty-seven patients (38.5%) underwent surgery after initial PCD. Indications for surgical intervention were ongoing sepsis not controlled by interventional radiologic management. In that study, percutaneous catheter drainage achieved sepsis reversal in 62% of patients and complete recovery was achieved without surgical intervention in 48% of patients[16].

Gagner first described minimally invasive surgical treatment of necrotizing pancreatitis in 1996, including laparoscopic retrocolic, retroperitoneoscopic, and transgastric procedures[71]. Over the past 15 years, several other minimally invasive surgical, endoscopic, and radiologic approaches for draining and debriding pancreatic necrosis have been described[23].

A literature search of the MEDLINE database from April 1996 to November 2010 was performed for each of the 4 techniques for minimally invasive necrosectomy: percutaneous therapy (341 studies), endoscopic necrosectomy (574 studies), laparoscopic necrosectomy via a transperitoneal approach (148 studies), and retroperitoneal necrosectomy (194 studies). Only cohorts with at least 10 or more patients were included. Twenty-seven studies with 947 patients were examined (8 studies on percutaneous approach; 10 studies on endoscopic necrosectomy; 2 studies on laparoscopic necrosectomy via a transperitoneal approach; 5 studies on retroperitoneal necrosectomy; and 2 studies on a combined percutaneous retroperitoneal approach). Finally, the authors advocated a multidisciplinary approach with interventional radiologists, gastroenterologists, intensivists, and hepatobiliary surgeons at tertiary care centers. They concluded that because the comparison data are limited, the minimally invasive approach should be based on location of lesion and individual patient presentation[23].

A prospective, randomized, multicenter trial called the Minimally Invasive Step Up Approach Versus Maximal Necrosectomy in Patients with Acute Necrotizing Pancreatitis (PANTER) was performed in the Netherlands[43]. After diagnosing necrotizing pancreatitis or infected pancreatic necrosis, patients were randomly assigned to either a step-up approach or 2 open necrosectomy. The step-up approach consisted of percutaneous drainage or endoscopic drainage, followed by a minimally invasive retroperitoneal necrosectomy if necessary. A video-assisted retroperitoneal debridement (VARD) with postoperative lavage was performed 3 d after if there was no clinical improvement. Major complications or death occurred in 31 of 45 patients after open necrosectomy (69%) vs 17 of 43 patients after the step-up approach (40%). About 35% of patients in the step-up group could be managed with percutaneous drainage only[43].

Similar to the PANTER Trial, there also is a recent, prospective multicenter, single-arm study from the University of Washington. Percutaneous drainage was used

as an initial treatment for infected pancreatic necrosis. If there was a 75% reduction in size based on a follow-up scan 10 d later, the remainder of their treatment would be percutaneous drains alone. If patients did not have a 75% reduction, they were treated with a VARD. Twenty-three percent of patients were treated with percutaneous drains only. Sixty percent of patients were treated with a minimally invasive intervention (*i.e.*, drains with or without a VARD). Mortality at 30 d was 2.5%. The percutaneous approach to infected pancreatic necrosis has been shown to be safe and feasible in multiple retrospective case series. It is noteworthy that 44% of patients reviewed in the studies did not need surgical therapy. What has become increasingly popular is combined percutaneous technique with a VARD as mentioned in the PANTER trial and the Horvath study[72]. These studies not only confirmed a subgroup of patients that can benefit from percutaneous drainage alone but also examined a combined technique in a prospective manner with a relatively larger amount of patients.

Retroperitoneal laparoscopic debridement drainage (RLDD) for treating retroperitoneal infected necrosis in SAP has been rarely reported, and there has been no report regarding comparison of curative efficacy between RLDD and laparotomy. This study showed that RLDD (a minimally invasive procedure) has obvious advantages for treating SAP retroperitoneal infected necrosis. It is safe and effective when done early and can prevent systemic inflammatory response syndrome from progressing further[22].

The overall message of these studies is that in patients who do not improve after adequate drainage, necrosectomy should be performed next. The percutaneous drain, together with the computed tomography scan, can be used as a roadmap for (minimally invasive) necrosectomy. Percutaneous (or transgastric) drainage should be the first intervention, and the indication for drainage should be the same as for surgical necrosectomy[3].

Direct endoscopic necrosectomy (DEN) is a minimally invasive treatment introduced recently for treating infected WON[73]. Using DEN, a stoma is created endoscopically between the enteric lumen and the walled-off fluid collection, allowing insertion of an endoscope into the fluid collection, which allows for an endoscopic necrosectomy. Current data suggest that DEN is a less invasive and less risky alternative to open surgical necrosectomy for managing infected WON and infected pseudocyst with solid debris[74].

Two large, multicenter, retrospective studies demonstrated that necrosis managed using direct transluminal endoscope techniques resulted in a positive prognosis and a high success rate at the beginning[75,76]. Nevertheless, all of the current endoscopic techniques have inherent limitations (*e.g.*, risk of air embolism, endoscopically uncontrollable bleeding, and inadequate drainage through multiple plastic stents) together with early occlusion of the fistulous tract. To overcome these difficulties, Hritz and associates demonstrated a successful method of endoscopic transluminal necrosectomy - a combination of the temporary placement of a self-expanding metal stent into the fistulous tract and daily irrigations of the necrotic cavity with a high-flow water-jet system using a flush knife[77].

Percutaneous techniques, including VARD, need open necrosectomy in a high proportion of patients, and mortality is around 20%[3]. It is now well recognized that most sterile collections do not require intervention (at least in the early phase of disease), and that mortality and morbidity rates after an intervention are time dependent, falling to almost 0% by the stage of a sterile WON. The indication for early intervention for infected necrosis is limited to sepsis control, and there is increasing consensus within this group that some form of minimally invasive approach may enhance outcomes. Conventional management of late pancreatic collections was by open pancreatic cystgastrostomy, but with developments in interventional radiology, therapeutic endoscopy, and minimal access surgery, new techniques have been used as alternatives to this approach[78]. While all have proven to be feasible in small cohort series, there is little evidence to the relative benefits of one method over another for managing APFC[79,80].

Laparoscopic cystgastrostomy (LCG) is used in mature symptomatic collections. It facilitates complete drainage of the collection with a minimal requirement for re-intervention. It also allows simultaneous management of gallstones. Laparoscopic cystgastrostomy should allow a wide debridement of the cyst cavity with the advantages of a minimally invasive approach. Open cystogastrostomy (OCG) is used when an intervention is required on additional intra-abdominal pathology (*e.g.*, enteric stricture or fistula) or where collection anatomy precludes other approaches. Laparoscopic cystgastrostomy allows larger collections to be managed by a one-step intervention, and the solid necrosis to be more effectively drained. Importantly, definitive management of gallstones can be achieved. However, the concept that endoscopic ultrasound (EUS)-guided drainage (the least invasive approach) may be of most benefit in fluid predominant collections requires evaluation within a study format, as experience has shown some APFC with significant necrosis may resolve completely using this approach only. Optimal management of collections with intermediate (size and fluid content) characteristics is not clear, and there may be clinical equipoise regarding whether a laparoscopic or endoscopic cystgastrostomy should be used as the preferred approach. A well-conducted, randomized, controlled study is required to determine which method is most effective in this particular group of patients[78].

In summary, standard treatment for infected pancreatic necrosis is open or laparoscopic surgical drainage. However, on occasions, percutaneous drainage may work well. As recommended by the International Association of Pancreatology Clinical Guideline, drainage should be effectively established when the patient is septic. A step-by-step treatment is proposed by which percutaneous or

endoscopic drainage should be established first and then necrosectomy with drainage through a minimally invasive retroperitoneal access. When this method was compared with open surgery, it offered several advantages including the chance to avoid surgery in some patients, fewer complications, and lower cost[43,53,70].

The alternatives to open surgery should be considered, mainly in frail and critical patients who would not tolerate more aggressive surgery. In clinical practice, it is important to consider the importance of a multidisciplinary management, considering the clinical situation and comorbidity of the patient and the experience of the personnel.

Pancreatic duct breaking: Generally this is produced in the context of pancreatic necrosis because of erosion of the duct. In cases of necrosis, complete or partial pancreatic duct breaking occurs in about 60% of cases. To assess this situation, wirsungography by using computed tomography, nuclear magnetic resonance (spectroscopy), or endoscopic retrograde cholangiopancreatography can be performed. This latter method may be associated with placing a stent, which will favor definitive resolution. Nutritional support and potent antisecretors (*e.g.*, octreotide) should be associated. Collections can be removed by percutaneous or endoscopic drainage. Successful fistula sealing is described using cyanoacrylate or fibrin[81]. If other treatments fail (which is common) surgery is indicated. However, in cases of complete duct rupture, it is rarely successful to access the residual duct in the pancreatic tail. In such cases, a distal pancreatic resection may be curative. Otherwise, internal drainage through a pancreaticodigestive anastomosis, may be necessary[1].

Pancreatic pseudocyst

According to several retrospective studies, the incidence of a pseudocyst after acute pancreatitis varies depending on the definition and methods of detecting a pseudocyst. The incidence ranges from 5% to 16%, and is reported as being higher in patients with underlying chronic pancreatitis[82-84].

Treatment of pancreatic pseudocysts: Fluid collections that appear during disappear spontaneously in 40% to 50% of cases. In about 10% to 15% of cases, these collections persist and become encapsulated, generating pancreatic pseudocysts. A true pancreatic pseudocyst (*i.e.*, without an epithelial lining; the counterpart would be a pancreatic cyst) takes at least 4 to 6 wk from the beginning of symptoms to be encapsulated by a wall formed by inflammatory fibrosis of the adjacent tissues. Few studies have documented the natural evolution of pancreatic pseudocysts. It has been thought that pancreatic pseudocysts more than 6 cm in diameter, or those that persisted for more than 6 wk, should be operated on[1] despite some studies showing that 50% of those which had no symptoms or were smaller than 10 cm resolved of their own accord[84]. It also has been shown that about

half of all pancreatic pseudocysts can be solved spontaneously; thus, the attitude has shifted toward a more conservative approach.

Asymptomatic pancreatic pseudocysts may be followed for periods of 6 mo or longer if they do not grow, become symptomatic, or present complications (*e.g.*, hemorrhage, infection, or mechanical compromise of adjacent organs). In these situations, percutaneous, endoscopic, or surgical drainage should be considered. It depends on several factors: patients' general status, size, number, and location of pseudocysts, communication (or not) with the main pancreatic duct, solid necrosis inside (or not), and possible complications[1].

Despite almost 50% of pseudocysts resolving themselves, the remainder can become symptomatic or infected, and may rupture, hemorrhage, develop vascular thrombosis, or obstruct nearby viscera, resulting in the need for some kind of medical intervention[85,86].

A ruptured pseudocyst, if it causes hemorrhaging in the digestive tract, will need immediate treatment, while if it occurs in the peritoneal cavity, it can lead to peritonitis or hemorrhagic shock requiring emergency exploratory surgery[25].

Kim *et al*[84] report spontaneous resolution, including disappearance and a size decrement, was achieved in 71.6% of cases despite the higher proportion of underlying chronic pancreatitis, and there was no significant difference in spontaneous resolution rate between acute and acute-on-chronic pancreatitis groups. Therefore, the wait-and-see policy for more than 4 to 6 wk may be feasible, unless the pseudocysts are associated with symptoms or complications. Although there have been differing results concerning spontaneous resolution of pseudocysts according to the study, size, detection time, and cause of the underlying pancreatic disease were reported as predictive factors[87-89]. The presence of an underlying chronic pancreatitis, an alcoholic cause, and a long interval from symptom onset until admission are risk factors for a pseudocyst, and a single lesion is a predictor of spontaneous resolution[84].

Percutaneous drainage should be avoided in cases of hemorrhage or pancreatic ascites. Surgical treatment (mainly by internal drainage) is reserved for patients in whom percutaneous or endoscopic treatment has failed, those with complications from chronic pancreatitis, those with multiple or giant pseudocysts, or when malignancy cannot be ruled out[90,91].

Hemorrhage or pseudoaneurysm

Hemorrhagic complications: Hemorrhagic complications of acute pancreatitis are fortunately rare; however, they may present in a diversity of forms. Sometimes, upper or lower gastrointestinal bleeding occurs because of gastroduodenitis secondary to adjacent inflammation, bleeding peptic ulcer, pseudocyst rupture into the digestive tract, or drainage of a pseudoaneurysm through the Wirsung duct. In severe cases of acute pancreatitis, bleeding may occur due to intra- or retroperitoneal erosion of

the vessels of the celiac trunk, mainly the splenic artery. Diagnosis may be established by angiography or angio-computed tomography. Angiography, besides identifying the bleeding point, sometimes allows embolization that may stop bleeding. If this method fails, definitive treatment must be surgery[92].

Ischemic complications (either local or related to remote vascular events) and venous or arterial complications - specifically splanchnic thrombosis and associated varices - are a major cause of morbidity and mortality[93]. The reported frequency of pulmonary embolism in acute pancreatitis is rare. The thrombohemorrhagic complications in pancreatitis play a tremendous part in developing its most severe forms and fatal outcomes. Early recognition and investigation of thromboembolism is imperative because accurate diagnosis and timely radiologic interventional procedures reduce mortality. Early treatment with intravenous heparin or thrombolysis is effective. Vascular filter insertion may be a life-saving measure for such patients[94].

Pseudoaneurysm

Pseudoaneurysm is a rare but potentially fatal complication of acute pancreatitis. The risk of rupture is as high as 37%[95]. The arteries involved include, in order of frequency, the splenic (40%), gastroduodenal (20%), pancreaticoduodenal (20%), gastric (5%), and hepatic (2%)[96]. The pathogenetic mechanism is secondary to degradation of the vessel wall by pancreatic enzymes released from a destroyed pancreatic duct, resulting in a primary formation of a pseudoaneurysm or rupture of the vessel into a pre-existing pseudocyst, which then converts into a pseudoaneurysm. Pseudoaneurysms present symptoms such as gastrointestinal bleeding (60%), abdominal pain (50%), and splenomegaly or pulsatile abdominal tumors (5%), and spontaneous regression also have been reported[97,98].

Generally developing intracystically, they are usually diagnosed *via* angiography, which is used for locating and treating with embolization (with a high technical success rate of 93%-100%, and low 24 h and 30 d re-bleeding rates - 4% and 17%, respectively, Kalva *et al*[99]), but it should also be borne in mind when a pancreatitis patient is undergoing a CT scan. Gonzalez *et al*[100] have also demonstrated that lipiodol with n-butyl cyano-acylate injected using endo-ultrasonography can be successful. If these techniques are not successful or if re-bleeding occurs, then surgery is required[25].

Necrotizing pancreatitis and pseudocysts involving the pancreatic tail appear to predispose patients to splenic complications[101]. The incidence of pseudocyst extension into the spleen has been estimated at around 1%. Erosion of noncystic pancreatic inflammation occurs less commonly[102,103]. In a series of 500 patients with chronic pancreatitis, splenic complications were found in 11 patients (2.2%), four of whom presented with splenic rupture. Five patients had intrasplenic pseudocysts and 2 had intrasplenic subcapsular hematomas[104]. A series of 159 CT scans performed on 100 consecutive

patients with acute pancreatitis found splenic infarcts in 10 patients and subcapsular hemorrhage in 2 patients[105]. Another series of 238 patients with pancreatic pseudocysts found 14 patients (5.9%) with splenic parenchymal involvement[106].

Management of patients with subcapsular hematomas and/or splenic parenchymal pseudocysts is by conservative approach, percutaneous drainage, or surgery[106]. The hemodynamically unstable patient with splenic rupture or hemoperitoneum requires emergency laparotomy and either splenectomy or distal pancreatosplenectomy, which can reduce the risk of pancreatic leak or fistula formation[104,106]. In hemodynamically stable patients, the decision for intervention should be based on clinical parameters rather than computed tomography imaging alone. A clinically stable patient with improving symptoms and resolving clinical signs can be managed conservatively with the intent of splenic conservation. Follow-up is by serial ultrasound or computed tomography scans, which can show spontaneous regression. Time for resolution varies from 1 wk to 4 mo depending on the severity of the underlying pancreatitis[107].

Chylous ascites

Pancreatitis is a rare cause of chylous ascites formation. It is believed that either lymph may actually leak through destroyed lymphatics because of pancreatic enzyme erosion or that chylous accumulation results from exudation of chyle, caused by the obstruction of lymphatic channel flow secondary to severe inflammatory changes that take place in the retroperitoneal space surrounding the pancreas[108]. Most cases involve chronic pancreatitis, though acute pancreatitis also has been recognized as the causative reason, with the first such report dating to 1984[109]. Since that time, only a few cases of chylous ascites secondary to acute pancreatitis have been documented. In almost all, the presence of chyle into the peritoneal cavity was discovered some time after the episode of pancreatitis, usually days or weeks[108]. However, Khan *et al*[110] reported a case of acute hyperlipidemic pancreatitis (with normal serum amylase) that presented with acute chylous peritonitis and was treated conservatively. Smith *et al*[111] operated on a patient with relapsing pancreatitis and acute chylous ascites formation caused by a clinical resemblance with appendicitis.

Therapeutic choices may vary in accordance with the underlying pathology.

Thorough lavage of the abdomen and adequate drainage is an excellent treatment modality for acute chylous peritonitis, because resolution of chylous ascites usually occurs within the next few days. However, successful conservative treatment also has been reported[112]. Conservative treatment requires proper preoperative diagnosis, which is often difficult because of the exceptional rarity of this condition and its resemblance to other surgical urgencies that call for immediate laparotomy. Long-term fasting, supported by total parenteral nutrition, frequently offers resolution. Alternatively, a high-protein low-fat diet

is effective at reducing the amount of chyle produced. Administration of octreotide is controversial[108].

In summary, the mortality rate for severe acute pancreatitis stands at between 15% and 30%, while if the between 5% and 10% of patients with parenchyma or peripancreatic necrosis are left untreated and it becomes infected, the mortality rate can be as high as 100%. The surgical methods and its timing are contentions regarding treatment of severe acute pancreatitis. Many studies showed that early surgery often was accompanied by higher mortality and morbidity rates. Faced with high morbidity and mortality rates of operative necrosectomy, minimally invasive strategies are being explored by gastrointestinal surgeons, radiologists, and gastroenterologists. In cases where there are severe acute pancreatitis complications, minimally invasive treatment is unsuccessful, or if there is widespread necrosis in locations not easily reached using other techniques, then traditional open surgery is strongly recommended.

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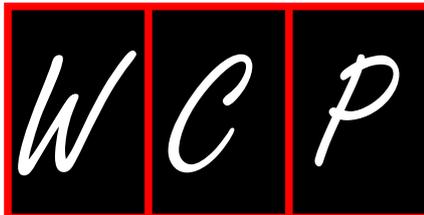
Treatment of severe acute pancreatitis and its complications

Enver Zerem

Enver Zerem, Department of Gastroenterology, University Clinical Center Tuzla, 75000 Tuzla, Bosnia and Herzegovina

Enver Zerem, Department of Medical Sciences, The Academy of Sciences and Arts of Bosnia and Herzegovina, 71000 Sarajevo, Bosnia and Herzegovina

Author contributions: Zerem E was the sole contributor to this paper.

Correspondence to: Enver Zerem, MD, PhD, Department of Gastroenterology, University Clinical Center Tuzla, Tuzla, Trnovac bb, 75000 Tuzla, Bosnia and Herzegovina. zerem@live.com

Telephone: +387-35303300   Fax: +387-35266485

Abstract

Severe acute pancreatitis (SAP), which is the most serious type of this disorder, is associated with high morbidity and mortality. SAP runs a biphasic course. During the first 1-2 wk, a pro-inflammatory response results in systemic inflammatory response syndrome (SIRS). If the SIRS is severe, it can lead to early multisystem organ failure (MOF). After the first 1-2 wk, a transition from a pro-inflammatory response to an anti-inflammatory response occurs; during this transition, the patient is at risk for intestinal flora translocation and the development of secondary infection of the necrotic tissue, which can result in sepsis and late MOF. Many recommendations have been made regarding SAP management and its complications. However, despite the reduction in overall mortality in the last decade, SAP is still associated with high mortality. In the majority of cases, sterile necrosis should be managed conservatively, whereas in infected necrotizing pancreatitis, the infected non-vital solid tissue should be removed to control the sepsis. Intervention should be delayed for as long as possible to allow better demarcation and liquefaction of the necrosis. Currently, the step-up approach (delay, drain, and debride) may be considered as the reference standard intervention for this disorder.

Core tip: This review reports on the natural clinical course, diagnostic possibilities and treatment modalities in severe acute pancreatitis (SAP). The management of SAP varies with the severity and depends on the type of complication that requires treatment. Although no universally accepted treatment algorithm exists, the step-up approach using close monitoring, percutaneous or endoscopic drainage, followed by minimally invasive video-assisted retroperitoneal debridement has demonstrated to produce superior outcomes to traditional open necrosectomy and may be considered as the reference standard intervention for this disorder.

INTRODUCTION

Severe acute pancreatitis (SAP) is associated with high morbidity and mortality due to the development of pancreatic and extra-pancreatic necrosis, their subsequent infection and multisystem organ failure (MOF)[1-3]. Despite overall reduced mortality in the last decade, SAP is a devastating disease that is associated with mortality ranging from less than 10% to as high as 85%, according to various studies[1-8]. The management of SAP is complicated because of the limited understanding of the pathogenesis and multi-causality of the disease, uncertainties in outcome prediction and few effective treatment modalities. Generally, sterile necrosis can be managed conservatively in the majority of cases with a low mortality

Severe acute pancreatitis

Week 1-2: SIRS   > 1-2 wk: Infections/sepsis
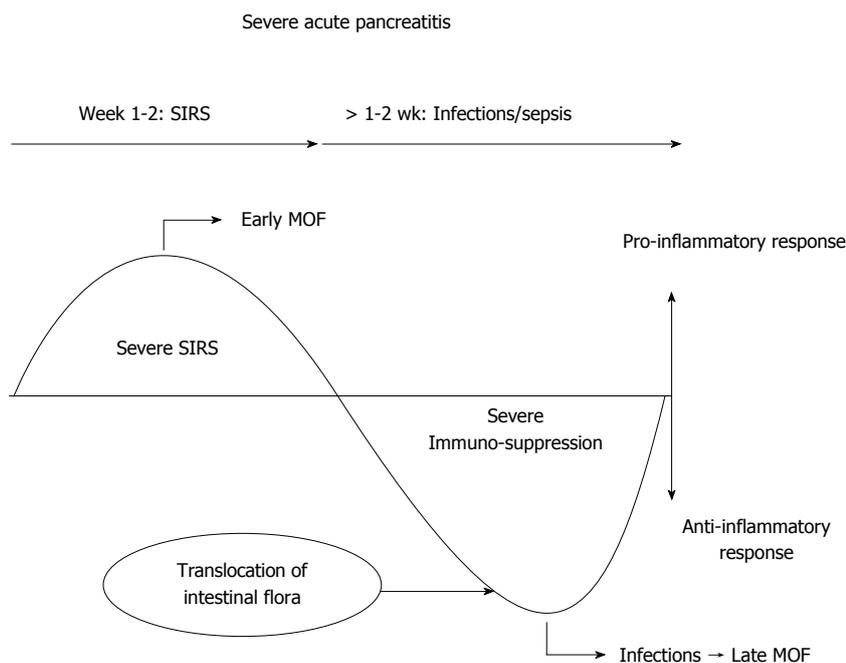
Figure 1 Natural clinical course of severe acute pancreatitis. SIRS: Systemic inflammatory response syndrome; MOF: Multisystem organ failure.

rate (12%)[2,9]. However, infection of pancreatic necrosis can be observed in 25%-70% of patients with necrotizing disease; it is generally accepted that the infected nonvital tissue should be removed to control the sepsis[1,10,11]. Laparotomy and immediate debridement of the infected necrotic tissue have been the gold standard treatment for decades[1,3,12]. However, several reports have shown that early surgical intervention for pancreatic necrosis could result in a worse prognosis compared to cases where surgery is delayed or avoided[2,3,6,8,13-17].

Therefore, several groups worldwide have developed new, minimally invasive approaches for managing infected necrotizing pancreatitis[2,3,6,18-24]. The applicability of these techniques depends on the availability of specialized expertise and a multidisciplinary team dedicated to the management of SAP and its complications[25].

NATURAL CLINICAL COURSE OF SAP

SAP develops in two phases (Figure 1). During the first 1-2 wk, a pro-inflammatory response occurs, which results in systemic inflammatory response syndrome (SIRS), a sterile response in which sepsis or infection rarely occurs. If the SIRS is severe, then proinflammatory mediators can cause early multiple (respiratory, cardiovascular, renal, and hepatic) organ failure. In parallel, pancreatic necrosis develops, usually within the first four days after the onset of symptoms. However, the extent of pancreatic necrosis is not fixed and may progress as the disease evolves during the first 2 wk[25]. Although in the early phase of severe pancreatitis, SIRS can be found in the absence of significant pancreatic necrosis, the majority of patients with severe early organ dysfunction will have

pancreatic necrosis that is evident on computed tomography scan[4,26]. Peripancreatic fluid collections are common and are termed acute fluid collections if present for less than 4 wk, after which time they are referred to as pancreatic pseudocysts (PPCs).

After the first 1-2 wk, a transition from a pro-inflammatory to an anti-inflammatory response occurs. During this "second or late phase", the patient is at risk for the translocation of intestinal flora due to intestinal barrier failure, which is followed by the development of secondary infection in the pancreatic or peripancreatic necrotic tissue and fluid collections. Mortality occurs in two peaks. Early mortality is the result of severe SIRS with MOF. Late mortality is the consequence of infection in the pancreatic necrosis and peripancreatic fluid collections resulting in sepsis[7,10,27,28].

DIAGNOSIS OF SAP

Diagnosis of SAP is based on clinical presentation, laboratory tests, and imaging results[29-34]. Physical and radiologic scoring systems have been developed with the aim of predicting which patients will have a severe clinical course and which patients might recover without major physiologic insult[32,34]. However, acute pancreatitis (AP) is a complex disease; despite the existence of several criteria, it is not easy to predict its subsequent course because often in patients with the same initial clinical and radiological scores, the clinical course of the disease may vary. It is difficult to assess the disease because of the lack of accurate and uniformly accepted definitions of disease severity and commonly encountered complications of AP[16,35,36].

Computer tomography findings	Grade	Score
Balthazar		
Normal pancreas	A	0
Enlargement	B	1
Inflammation of pancreas and fat	C	2
Single fluid collection	D	3
Two or more fluid collections	E	4
Necrosis		
< 30%		2
30%-50%		4
50%		6
		Max = 10 points

Table 1 Computer tomography indeks of illness severity for acute pancreatitis[48]

Clinical and laboratory investigations

During physical examination, the most common presenting symptoms of AP are epigastric pain, nausea, and vomiting with physical signs that can include rebound tenderness, distension and reduced bowel sounds. Systemic involvement and organ failure can be detected, including shock, pulmonary insufficiency, renal failure, gastrointestinal bleeding or any combination of these symptoms[37-39].

Laboratory findings in SAP usually reflect organ dysfunction and metabolic disturbances. Used for diagnosing AP, serum amylase and lipase levels greater than three times the upper normal limit is considered to be diagnostic of pancreatitis. In AP, these enzymes are elevated because of the pancreatic acinar cell leakage into the interstitial space and their subsequent absorption into the circulation[40].

There are various scoring systems (Ranson, APACHE Ⅱ, SOFA, BISOP, *etc.*) that help stratify the severity of AP. The severity of AP which can be objectively assessed on the patient's admission to the hospital by using Ranson's score[33], or the APACHE Ⅱ criteria for disease severity[41], which evaluate the disease severity based on laboratory and clinical parameters. During the course of AP, the disease is considered to be severe if 3 or more Ranson's criteria are observed within 48 h of the onset of the attack, or if 9 or more APACHE Ⅱ criteria are observed at any time during the course of the disease. The severity of organ failure, determined using SOFA score multi-step criteria, as introduced for septic patients, is considered to be clinically relevant and is being increasingly applied for scoring disease severity and for predicting outcome[42]. The bedside index for severity in AP (BISAP) is a simple clinical scoring system, which stratifies patients within the first 24 h of admission to the hospital according to their risk of in-hospital mortality and helps identify patients at increased risk for mortality before the onset of organ failure. A score of > 3 is associated with 5%-20% mortality[43,44].

However, as in other disease processes, physicians face numerous dilemmas in defining AP severity and its complications. To help physicians define AP, a multidisciplinary International Symposium was organized in Atlanta in September 1992 with the aim of achieving international consensus on the definition of AP and its complications[45]. Despite the worldwide acceptance of The Atlanta Classification as the first reliable clinical classification system of AP, the accumulation of clinical data calls for a revision of the Atlanta criteria of severity[46].

Imaging evaluation

Contrast-enhanced computed tomography (CECT) is currently the standard imaging modality in the setting of SAP. The most important roles for CECT are the diagnosis of pancreatic gland necrosis, the determination of the extent of necrosis, and the diagnosis of local complications[25,47]. Because the complete development of pancreatic necrosis may not occur for up to four days after the onset of SAP in the majority of patients, CECT cannot be used to reliably determine the presence or the full extent of necrosis before that time[9]. CECT cannot reliably detect underlying necrotic debris in an acute necrotic collection or walled-off necrosis (WON), especially fluid-predominant collections[25,47]. The Balthazar's CT severity index (CTSI)[48] is commonly used to stratify the severity of the disease and to predict mortality (Table 1).

Ultrasound (US) has a limited role in the assessment of patients with AP; its primary disadvantage is the frequent association with the ileus, which tends to make the visualization of the pancreas difficult[49]. Another disadvantage of US is that it provides no information regarding the presence or the extent of pancreatic necrosis. However, compare to the majority of other modalities, the primary advantage of ultrasound is that it is a portable procedure that can be performed in any location, which is especially useful with for patients who are in a critical care setting and who cannot be easily transported to the CT scan suite.

Endoscopic ultrasound (EUS) is a useful modality for evaluating patients with AP. Its role in the assessment of choledocholithiasis is to aid in triaging patients who require therapeutic endoscopic retrograde cholangiopancreatography (ERCP), thus eliminating potential complications that might be associated with diagnostic ERCP. The limitations of EUS are the inconsistent availability of skilled endosonographers with endoscopic and imaging skills, a potential for adverse events in critically ill patients, and a tendency to overestimate the necrotic debris content of pancreatic fluid collections[25,49].

Magnetic resonance imaging (MRI) is a good alternative to CT for detecting parenchymal necrosis; magnetic resonance cholangiopancreatography (MRCP) may replace ERCP in the diagnostic evaluation of the pancreatic duct (PD)[47,50-52]. Due to its ability to characterize pancreatic and peripancreatic collections or abscesses as partial or full fluid in consistency, lack of radiation, ability of MRCP to detect bile duct stones, and ability to demonstrate the presence of disconnected PD, MRI has a fundamental impact on the course of additional management. Disadvantages of MRI/MRCP include longer acquisition times, difficult implementation in critically

Table 2 Principles of intensive monitoring and systemic support

Parameters
Intensive invasive monitoring of vital constants
Analgesics (consider epidural analgesia if necessary)
Fluid resuscitation with monitoring of central venous pressure
Electrolyte solutions
Plasma expanders
Humidified oxygen administration
Catecholamines (dopamine, dobutamine)
Early nutritional support
Early treatment of systemic complications
Mechanical ventilation with positive end-expiratory pressure
Catecholamines (epinephrine)
Hemofiltration, dialysis
Insulin and calcium substitution

ill patients, toxicity of gadolinium in patients with renal insufficiency, and contraindication of MRI in pacemakers and other metal objects[25,49-52].

Image-guided, fine-needle aspiration of the necrotic area is a procedure used for obtaining culture and Gram stain and identifying the causative organism of infection. However, therapeutic trends have altered this approach to such a degree that the clinical relevance of this method has been substantially diminished[25].

BASIS OF THERAPY IN SAP

SAP should be managed in an intensive care unit that is equipped to apply intensive monitoring and systemic support, including supportive care, prompt fluid resuscitation to maintain circulation volume and prevent electrolyte imbalance, nutritional supplements, analgesics, oxygen supplementation, mechanical ventilation, as well as monitoring for respiratory, cardiovascular and renal insufficiency and their early correction[3,7,53-55]. The principles of intensive monitoring and systemic support in SAP are summarized in Table 2.

There are two primary aims in the initial treatment of patients with SAP. The first aim is to provide supportive therapy and to treat specific complications that may occur. The second aim is to limit both the severity of pancreatic inflammation and necrosis and SIRS by specifically interfering with their pathogenesis[1]. The clinical usefulness of protease inhibitors (somatostatin, octreotide, lexipafant and gabexate mesilate) in the treatment of SAP has not been clearly confirmed despite the fact that several studies have shown a reduced incidence of complications and mortality after the administration of protease inhibitors[56-58]. Thus, the conservative treatment of AP is still primarily symptomatic and the specific medication that affects the cause of the disease is not currently available.

Nutritional support

SAP is characterized by marked nutritional depletion, and nutritional support is required to achieve a positive nitrogen balance. Because these patients may often pres-

ent with paralytic ileus and keeping the pancreas at rest is mandatory, the patients are parenterally fed. Parenteral nutrition should be started, and positive nitrogen balance should be obtained in the first 72 h after the onset of SAP. Enteral nutrition starting in the early phase of SAP is superior to total parenteral nutrition unless paralytic ileus is present[59]. This positive effect is most likely achieved using enteral nutrition that supports maintenance of the intestinal barrier. Continuous tube feeding with peptide-based formulae is possible in the majority of patients, and the jejunal route is recommended if gastric feeding is not tolerated by the patient. If the volume of enteral nutrition tolerated by the patient is insufficient to achieve adequate caloric support, combined parenteral and enteral feeding should be instituted[60].

Role of antibiotics

The aim of antibiotic prophylaxis in SAP is to prevent superinfection in the necrotic tissues. Late deterioration of organ dysfunction, which occurs most commonly between the second and third week after the onset of SAP[61], most likely results from secondary infection in pancreatic and peripancreatic necrosis due to bacterial translocation from the gastrointestinal tract into the necrotic tissues. Because the development of necrosis is currently not preventable, the rationale for using prophylactic antibiotics in SAP is to prevent the infection in the pancreatic necrosis[1]. However, antibiotic prophylaxis is controversial concerning the clinical management of AP. There are a large number of published studies with questionable study designs and contradictory results, which could be attributed to the inclusion of heterogeneous patients, different antibiotic regimes, and different study objectives[54]. Several randomized controlled trials offer evidence for the effectiveness of prophylactic antibiotics in reducing septic complications and mortality of patients with necrotizing pancreatitis[62,63]. However, other studies, of which several are meta-analyses, as well-designed studies, don't approve the routine use of prophylactic antibiotics because there are no significant differences related to surgery or mortality. Two randomized, double-blinded, prospective, controlled, multicenter trials proved antibiotic prophylaxis to be ineffective concerning the reduction of infection in necrosis and hospital mortality[64,65]. A Cochrane meta-analysis concluded that antibiotic prophylaxis is not protective in SAP[66]. The American Association of Gastroenterology recommends the administration of antibiotic prophylaxis in cases of extended necrosis involving more than 30% of the gland based on abdominal CT. Prophylaxis should be administered for no longer than 14 d because prolonged antibiotic therapy increases the prevalence of fungal infections. The role of prophylactic antifungal agents has not been fully defined[54,64].

Treatment of biliary etiology

Although there is no clear consensus on all of the indications for ERCP and endoscopic sphincterotomy (ES),

Figure 2 Three catheters inserted percutaneously into the abscess collections formed during the clinical course of necrotizing pancreatitis.

it is generally accepted that they are indicated for acute cholangitis and obstructive jaundice[67,68]. Under these conditions, ERCP and ES ameliorate the symptoms and the progression of the disease when applied early, desirably within 72 h from the onset of the disease[69]. The question remains whether patients classified as suffering from severe biliary pancreatitis but without associated biliary sepsis or obstructive jaundice would benefit from the endoscopic approach. Open cholecystectomy is an unacceptable emergency procedure in patients with severe gallstone-associated pancreatitis. Co-morbidity, which is a major predeterminant of cholecystectomy outcome, does not apply to the use of ERCP and ES. Generally, patients with AP of suspected biliary etiology and who are classified as suffering from severe disease should undergo ERCP. ES should be performed when there is biliary sludge or stones within the common bile duct[70].

IMAGING-GUIDED AND ENDOSCOPIC PROCEDURE FOR TREATMENT OF NECROTIZING PANCREATITIS

Image-guided percutaneous treatment
Image-guided percutaneous interventions, which seem technically feasible in a vast majority of patients with necrotizing pancreatitis, range from needle aspiration to the placement of multiple drainage catheters[2,3]. The choice of image-guided intervention for percutaneous needle aspiration or percutaneous catheter drainage (PCD) depends on the size and the location of the collection and the patient's habitus[16,71].

Image-guided PCD of collections in and around the pancreas in patients with acute necrotizing pancreatitis is an important therapeutic option either on its own or as an adjunct to surgery. The majority of pancreatic collections are located in the lesser sac, the anterior pararenal space, or other parts of the retroperitoneum and can be drained with a catheter inserted percutaneously[3,16,49,72]. Moreover, the advantages of PCD include widespread availability, access by transperitoneal and retroperitoneal approaches to the left and right sides of the abdomen and pelvis,

the ability to insert multiple catheters (Figure 2), and the ability to flush catheters between procedures without general anesthesia and with fewer traumas, simultaneously performing vigorous irrigation with similar effects as performed surgically[3,73,74].

Depending on the operator experience, tandem trocar technique or Seldinger technique can be used. If the Seldinger technique is used, then the catheter tract should be sequentially dilated over a guidewire. Access routes that avoid crossing the bowel and other intervening organs, or major mesenteric, peripancreatic, or retroperitoneal blood vessels are selected to minimize the risk of bacterial contamination and hemorrhage. Successful percutaneous treatment of necrotic collections of the pancreas depends on several important factors. Catheters often need to remain in place for several weeks and sometimes months; hence, close follow-up is required[3,49,72].

The value of drainage therapy for removing solid debris is equivocal. Generally, at the beginning of the disease, catheter drainage of the infected necrotic tissue is poor; several authors have considered that surgical resection of the necrotic tissue is mandatory[1,7,9,11,12,14]. However, other authors have determined[2,5,6,16-21] that solid tissue and necrotic debris could be removed with draining fluid and that the use of vigorous irrigation through large-bore catheters could effectively remove the tissue. The rationale for this strategy is that large-bore catheters may be more effective for mobilizing solid tissue and evacuating the necrotic tissue from the cavities. Other authors have reported no significant correlation between the drainage catheter size and the disease outcome[3,8,16]. Several percutaneous drainage procedures are performed to stabilize the seriously ill patient before surgical debridement, whereas other procedures are performed with the intent to cure[38,72]. In 1998, Freeny et al[75] first described a consecutive series of patients who had infected pancreatic necrosis and who were treated primarily with imaging-guided PCD, as an alternative to primary surgical necrosectomy. They demonstrated that the majority of patients could be treated by drainage without the need for necrosectomy. A major limitation of PCD is the development of pancreaticocutaneous fistulae; several authors reported that several fistulas did not close after the procedure because of communication between the drain and an upstream disrupted PD[2,25]. However, the disruption of PD is the initial pathologic event that triggers fistula formation in inflammatory disease and trauma of the pancreas[76]. Therefore, the recovery of disrupted PD has been recognized as the primary prognostic factor for successful treatment of pancreatic fistula regardless of the treatment method (surgery or imaging intervention) used. Moreover, in several cases, the fistula can be successfully treated by image-guided PCD with irrigation by antiseptic and administration of proper antibiotics[77].

Endoscopic treatment of SAP
Endoscopic necrosectomy is a minimally invasive method

Table 3 Surgical treatment modalities in necrotizing pancreatitis[86]

Surgical treatment modalities
Open necrosectomy with open packing - after necrosectomy, the abdomen maybe left open and repeatedly debrided until there is no residual necrosis, and is allowed to close by secondary intention
Open necrosectomy with closed packing - after the removal of necrotic tissue, the abdomen is closed, packing with external drains left in place. The drains are removed singly every other day, starting 5-7 d postoperatively
Open necrosectomy with continous postoperative lavage - the procedure is based on the insertion of 2 or more double lumen catheters. Repeated open necrosectomy is performed and the packing is removed when there is no residual necrosis. The smaller lumen of the drains is used for the inflow of the lavage, and the larger lumen is used for the outflow. The drains can be removed after 2-3 wk
Programmed open necrosectomy - necrosectomy of necrotic tissue is performed using multiple procedures. After necrosectomy, the pancreatic bed is packed with sponges and soft drains are placed on the top of the packs. The abdomen is closed using a zipper

for the drainage of symptomatic pancreatic collections and necroses whereby a nasocystic catheter is inserted through a transmural entry site alongside a 10-Fr stent to perform irrigation. Endoscopic necrosectomy was first described in 1996 by Baron *et al*[78], whereas peroral flexible endoscopic drainage of PPCs performed *via* transpapillary or transmural techniques had been reported more than 25 years ago[25]. Using the direct endoscopic necrosectomy technique, a stoma is created endoscopically between the enteric lumen and the necrotic cavity to allow the insertion of an endoscope directly into the cavity, which allows mechanical debridement and lavage. Direct endoscopic necrosectomy can be performed only if the collection or necrosis is located within a few centimeters of the gastric or duodenal lumen. The site of transmural puncture for direct endoscopic intervention should be determined visually and fluoroscopically by an observed bulge that represents the extrinsic compression of the collection into the gut lumen. Approximately 50% to 80% of potentially drainable collections can be performed using this approach. However, a bulge is often absent with smaller collections, low serum albumin, and collections in or near the pancreatic tail[79-82].

Therefore, to minimize the risk of complications, such as puncturing adjacent structures, bleeding, and perforation, EUS is increasingly used to perform endoscopic drainage. The advantages of EUS-guided endoscopic drainage include the ability to visualize and determine the optimal access into the collection, to avoid intervening blood vessels, to assess the contents of the cavity, and to visualize bleeding into the collection and other complications during and immediately after the procedure[25]. Randomized clinical trials of endoscopic transmural drainage with and without EUS guidance showed that EUS visualization had an advantage over conventional endoscopic techniques[79,80].

The advantages of the endoscopic approach compared to PCD include internal drainage and avoidance of external fistulae; however, limitations include the need for multiple repeated procedures under sedation or anesthesia[25]. Additionally, in the case of superinfection or drainage problems, monitoring, catheter manipulation and analysis of cystic content are difficult using the endoscopic approach[49,83]. Combining a percutaneous approach and endoscopic transmural drainage can prevent external fistulae and avoid repetitive endoscopic interventions to perform direct necrosectomy[84].

SURGICAL APPROACH TO NECROTIZING PANCREATITIS

Open surgical necrosectomy

The indication for surgical intervention and the optimal timing of intervention in necrotizing pancreatitis are frequently subject to discussion[85]. Traditionally, laparotomy and immediate surgical debridement have been the gold standard for the treatment of infected and symptomatic sterile necroses with the aim of complete removal of the necrotic tissue[1,12,45]. Open necrosectomy, originally described by Beger *et al*[61] consists of a laparotomy through a bilateral subcostal incision. After blunt removal of all of the necrotic tissue, two large-bore drains for postoperative lavage are inserted, and the abdomen is closed.

Currently, there are various open surgical approaches for removing the pancreatic necroses. Table 3 outlines various strategies for open surgical necrosectomy[86].

Open necrosectomy is associated with a high morbidity (34%-95%) and mortality ranges from 6% to 25%[25]. Randomized controlled trials have demonstrated that delayed surgical necrosectomy proves superior to early necrosectomy[14]. Therefore, the current recommendation is to delay the surgery as late as possible after the onset of pancreatitis until the necrotic process has stopped expanding and when there is a clear demarcation between viable and nonviable tissues, so that the infected necrosis has become walled off or organized[9,69,86]. Potential, immediate, postoperative, adverse events include organ failure, perforation of a hollow viscus, wound infection, and hemorrhage, any of which may require another surgery. Long-term adverse events include chronic pancreaticocutaneous and enterocutaneous fistulae, diabetes mellitus, exocrine pancreatic insufficiency, and abdominal wall hernias. Consensus supports the claim that postoperative continuous irrigation and "closed packing" are superior to open packing and planned relaparotomies. Relaparotomy increases the local intra-abdominal and systemic trauma and has negative systemic effects on hemodynamic and systemic inflammatory response[25].

Minimally invasive surgical techniques

The traditional limitations of open surgery (significant postoperative deterioration and organ dysfunction) have led to the development of minimally invasive necrosectomy techniques as less invasive treatment alternatives to open necrosectomy[22]. They can be classified according to the type of scope used (laparoscope, nephroscope)
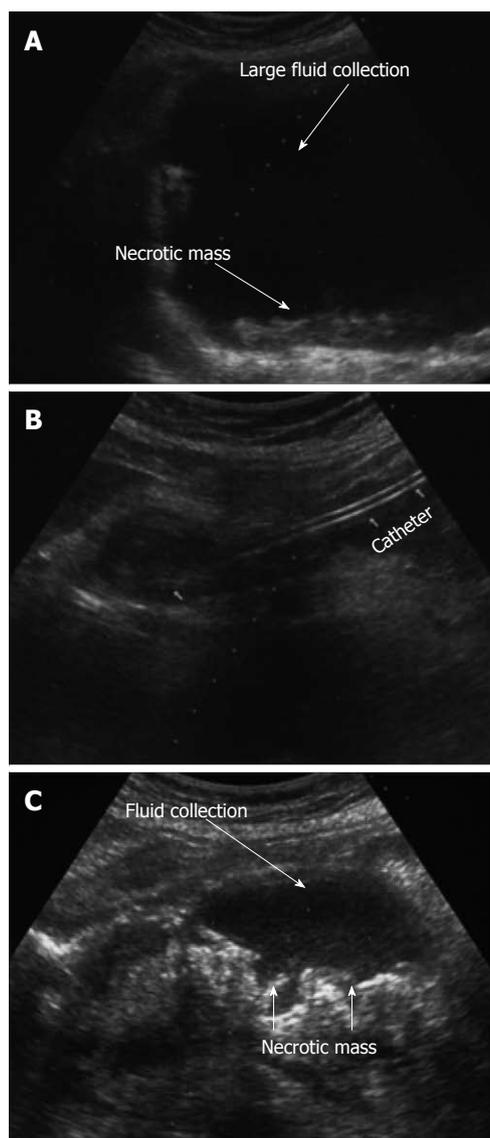
Figure 3 Ultrasound appearance of pancreatic necroses and a large acute fluid collection before and after drainage. A: Large fluid collection and pancreatic necroses before drainage; B: Catheter in the peripancreatic fluid collection; C: Massive pancreatic necroses with secondary fluid collection.

and the route of access (transperitoneal, retroperitoneal)[87-89] with the aim of minimizing the surgical stress and physiological insult in patients who are already critically ill[1,90,91]. Carter et al[92] described their technique and good results from percutaneous retroperitoneal necrosectomy. The retroperitoneal approach may be selected in patients with left-sided, predominantly retroperitoneal necrosis with a semisolid collection. In 2001, Horvath et al[93] described the video-assisted retroperitoneal debridement (VARD) approach, using a 4-5 cm retroperitoneal incision and regular laparoscopic equipment for removing the infected necrosis. Critics of these techniques noted that they require several repeated procedures to perform complete necrosectomy with a likelihood of serious complications. Each access route has its own advantages and disadvantages, such as ease of access, ability to address multiple collections and risk of collateral injury. The ac-

tual status of endoscopic drainage seems to differ only slightly from that of the percutaneous techniques[22].

MANAGEMENT OF COMPLICATIONS OF SAP

Management of complications of AP varies depending on the severity and the type of complications. Considering the Atlanta classification system is an important step before determining the strategy for treating the complications of AP because different local complications should be treated in different ways, either conservatively, using interventional methods, or surgically[45,46]. Treating the complications of SAP, including pancreatic fluid collections, necrosis, pseudocysts, abscesses, pancreatic fistulas, and hemorrhage, requires a multidisciplinary approach and the application of diagnostic, interventional and surgical methods.

AP fluid collections and necroses
Pancreatic necrosis develops early in the course of SAP and is usually well established by 96 hours after the onset of clinical symptoms. Acute necrotic collections, which occur simultaneously in approximately 40% of patients, as enzyme-rich pancreatic juice collections can be intrapancreatic or extrapancreatic. They are heterogeneous, can contain non-liquid material with varying amounts of fluid, and are without full encapsulation[38,45,49,72]. Sterile acute necrotic collections rarely require intervention early in the course of disease, and the conservative approach and image-guided follow-up of acute sterile fluid collections and necroses are better than continuous drainage from the beginning, which is frequently associated with their bacterial colonization and catheter problems[25].

However, several patients with gross destruction of the pancreatic gland due to impairment of the microcirculation of the pancreas during SAP can develop massive sterile pancreatic necroses, which cause systemic release of numerous cytokines and inflammatory mediators, thus leading to activation of inflammatory cells, fever, and multiorgan failure (Figure 3).

Although sterile pancreatic necroses are not infected, they can lead to extravasations of amylase-rich and protein-rich intravascular fluid into the peripancreatic regions and can result in poor clinical course and initiate physiologic pathways, which progress to organ failure, cardiovascular collapse and formation of abscesses and sepsis[16]. Therefore, in this clinical setting, the removal of toxic mediators and inflammatory substances from sterile collections may ameliorate the systemic consequences induced by SAP[16,94]. Removing toxic mediators and inflammatory substances can be performed by percutaneous or endoscopic transmural drainage[16,82,94,95].

Infected pancreatic necrosis
More than 80% of deaths associated with AP are attributed to septic complications as a consequence of bacterial infection in pancreatic necrosis[49]. Therefore, in
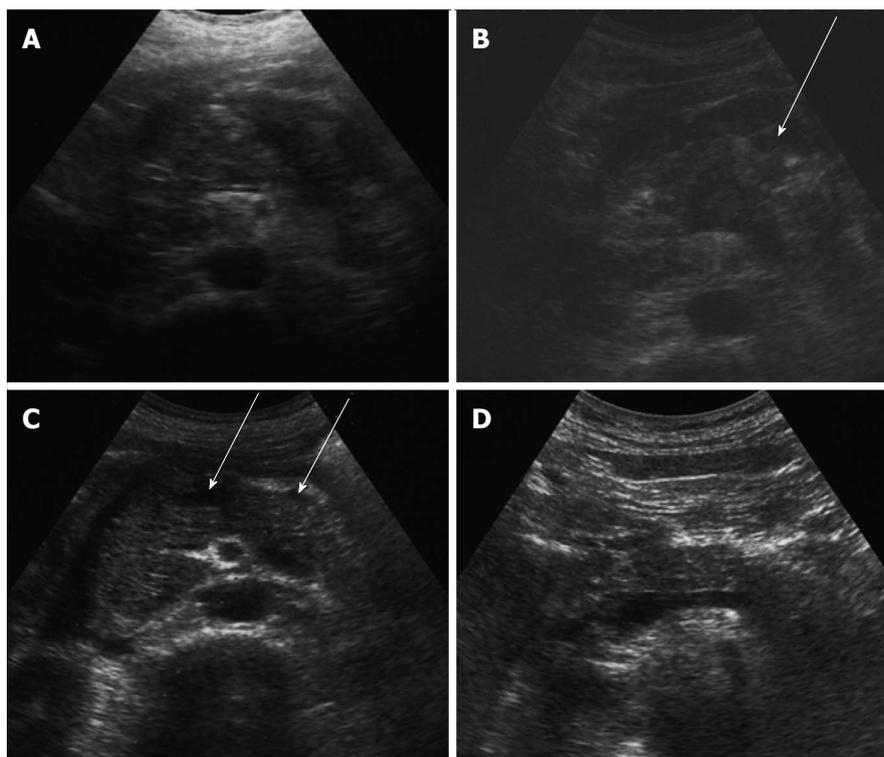
Figure 4 Ultrasound appearance of infected pancreatic necrosis before and after the treatment of acute pancreatitis. A: Infected pancreatic necrosis (IPN) involved the entire pancreas in the beginning of the disease; B: Liquefied areas in the IPN marked by arrows; C: Small necroses and liquid collections around the pancreas 2 mo after the beginning of treatment marked by arrows; D: Normal appearance of the pancreas 6 mo after the beginning of treatment.

infected necrotizing pancreatitis, the infected non-vital tissue should be removed to control the sepsis. For patients with infected necrosis, there is convincing evidence that the early surgical intervention (before 3 wk) for pancreatic necrosis could result in a worse prognosis compared to patients where surgery is delayed. Surgical techniques are associated with total anesthesia and considerable trauma, which often causes escalation of multiorgan failure, uncontrolled bleeding and sepsis[2,3,6].

Recently, minimal invasive non-surgical management, using truly conservative or less invasive drainage techniques, was included in the treatment of infected necrotizing pancreatitis that allowed for the surgical debridement to be delayed or completely avoided[2,3,6,14,15]. In the beginning of the disease, catheter drainage of infected necrotic tissue is often ineffective because of catheter blockage by necrotic tissue fragments and viscous fluid. However, during the course of SAP, a transition from solid necrotic tissue to more liquid contents leads to a higher success rate of the evacuation of the necrotic tissue from the cavities, regardless of the catheter size (Figures 4 and 5A)[3,19].

Therefore, conservative treatment with proper intravenous hydration and the administration of proper antibiotics should be performed at the initial stages of the disease. Less invasive drainage techniques should be considered when truly conservative treatment fails to resolve the infected pancreatic necrosis. Surgical necrosectomy may represent overtreatment at the beginning of the dis-

ease onset in patients with usually poor general condition, with difficulties in discriminating between necrotic and normal tissue during the procedure. Additionally, surgical necrosectomy carries a high risk of bleeding from vessels in the necrotized tissue during or immediately after the intervention. With delayed intervention, demarcation between the necrotic and vital tissue occurs; therefore, if necrosectomy is performed later in the course, then resection of the vital tissue is minimized, leading to better long-term endocrine and exocrine function and a reduction in postoperative adverse events[3,19,69].

Pancreatic WON

According to the revision of the Atlanta classification, pancreatic *WON* is defined as "a circumscribed collection of pus, containing little or no pancreatic necrosis, which arises as a consequence of AP or pancreatic trauma"[46,96]. *WON*, which occurs only in the context of necrotizing pancreatitis, is heterogeneous, contains non-liquid material with varying amounts of fluid, and has an encapsulating wall (Figure 5B).

WON can be located intrapancreatically or extrapancreatically. This process develops due to liquefaction and subsequent superinfection of limited pancreatic and retroperitoneal necrosis as well as superinfection of acute fluid collections[97-99]. In general, pancreatic WON develops later in the course of the disease (usually after four or more weeks after the onset of SAP). Asymptomatic WON does not mandate intervention, regardless of the
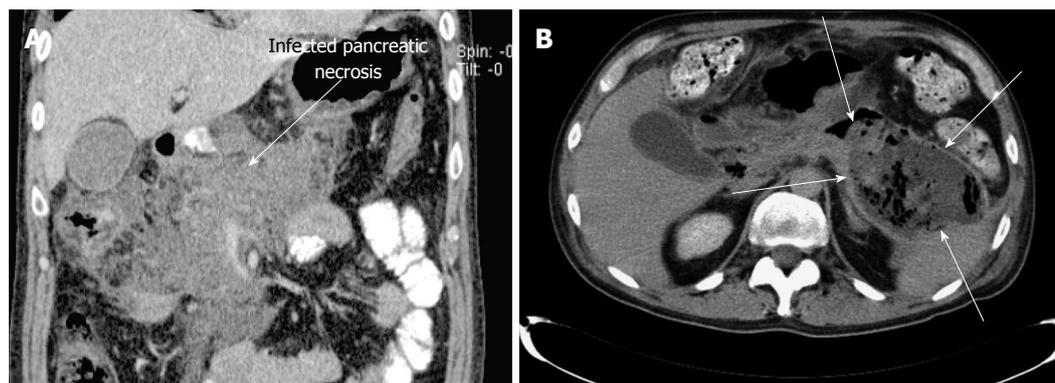
**Figure 5 Computed tomography.** A: Computed tomography (CT) appearance of the infected pancreatic necrosis, which involves the entire pancreas (marked by an arrow); B: CT appearance of a large pancreatic walled-off necrosis in the tail of the pancreas (marked by arrows).

size and extension of the collection, and may resolve spontaneously over a period of time, even in rare cases of infected necrosis[25]. Symptomatic WON generally requires intervention later in the disease course if there is intractable pain, obstruction of the stomach or bile duct, or in the case of infection[25,99,100]. Due to their less aggressive behavior and circumscribed localization, minimally invasive treatment strategies, including percutaneous or endoscopic approach, can be easily performed with success in the majority of these cases[99,101].

PPC
A PPC is a collection of pancreatic content enclosed by a wall of fibrous or granulation tissue, which is not lined by the epithelium[45]. The majority of PPCs regress spontaneously and need no treatment, whereas some PPCs may persist and progress to produce complications. Factors that influence the decision regarding whether to treat PPC include pain, infection, pressure effects that can lead to gastric outlet, intestinal or biliary obstruction. Several conditions must be met to achieve the complete obliteration of the cyst cavity. PD anatomy is an important factor in the prognosis of the treatment[76,83,102-104].

Traditionally, surgery was the only treatment option for symptomatic PPC[83,105]. However, this surgical treatment involves considerable trauma and general anesthesia, with the risk of PPC recurrence not being entirely excluded. The recent trend in managing symptomatic PPC has been toward less invasive approaches, such as endoscopic and image-guided PCD[83,102-104,106]. The endoscopic approach is suitable because the majority of PPCs lie adjacent to the stomach, yet with both endoscopic and imaging skills being required here[83]. The major advantage of the endoscopic approach is that it creates a permanent pseudocysto-gastric tract with no spillage of pancreatic enzymes. However, with drainage problems, monitoring, catheter manipulation and the analysis of cystic content are difficult or impossible to perform endoscopically, unlike with PCD approach[83]. Drainage techniques have better results and lower recurrence rates in patients without communication between PPC

and PD[76]. When PPC-PD communication is identified, the mean duration of drainage increases to between weeks and months, depending on the condition of the PD[76,103,104].

Pancreatic fistula
Disruption of the PD secondary to pancreatic necrosis leads to leakage of the pancreatic secretion and its accumulation inside the abdomen in the neighborhood of the pancreas and pseudocyst formation. However, the pancreatic juice can also flow to other locations, causing pancreatic ascites, pleural effusion, distant pseudocyst or pancreatocutaneous fistula. ERCP, MRCP and wirsungraphy by using CT may be utilized in the diagnostic evaluation of PD disruption[1,47,50-52,54]. ERCP, in the same endoscopic session, may be associated with the placement of a stent to bridge the leak site, which may contribute to the definitive resolution of PD disruption[107]. Traditionally, pancreatic fistulas have been managed primarily by conservative treatment with total parenteral nutrition and the administration of pancreatic secretory inhibitor octreotide. However, conservative treatment tends to fail in many patients whereby interventional therapies and even surgery become the next option. A subsequent surgery for fistula management is technically demanding and is associated with major morbidity and mortality[77,106-109].

Hemorrhage
Hemorrhage into the pancreatic bed or adjacent retroperitoneum is usually a consequence of gastrointestinal bleeding, which occurs due to gastroduodenitis, bleeding peptic ulcer and pancreatitis-induced enzymatic damage to the adjacent vasculature, such as the splenic, renal or gastroduodenal arteries and the development of an aneurysm in one of these arteries[38,45]. Rupture of an aneurysm in these arteries usually results in acute, severe, life-threatening hemorrhage. Diagnosis may be established by angiography or angio-CT. Occasionally, emobilization can be performed using angiography, which may stop the bleeding. If this method fails, the definitive treatment must be surgery[49,54,110].

STEP-UP APPROACH

In recent years, the treatment of infected necrotizing pancreatitis has shifted from early surgical necrosectomy to postponed minimally invasive step-up strategy. This approach is based on the statement that surgical debridement may represent overtreatment at the beginning of the disease in patients with usually poor general condition, with difficulties in discriminating between necrotic and normal tissue during the procedure and a high risk of bleeding from vessels in the necrotized tissue during or immediately after the surgery. The initial step-up approach is percutaneous or endoscopic drainage of the infected collection to prevent sepsis. If this approach fails, minimal invasive surgery is employed, with open surgery being reserved for those patients who do not respond to less invasive techniques[3,13,18,20,22-24,85,111].

If the patient's condition improves (in approximately 35% of cases)[22], after percutaneous or endoscopic approach, no surgical debridement is performed. Surgical intervention is postponed for as long as possible so that the infected collection may become encapsulated[3,22] and is performed when the patient's condition does not improve or if it deteriorates. Several recently published studies compared the outcomes of the step-up approach with open debridement as the primary treatment (step-down approach) and demonstrated that the step-up approach was superior because it reduced morbidity, mortality and costs per patient[3,6,13,18,20]. Presently, the step-up approach may be considered the reference standard intervention for SAP. The individual components of the step-up approach may be subject to improvement. However, the concept of the step-up approach can be summarized as follows: delayed intervention with close monitoring and conservative treatment, catheter drainage and minimally invasive drain-guided debridement seem here to stay[22].

CONCLUSION

The management of SAP varies depending on the severity and the type of complication that requires treatment. Classifying the complications of SAP according to the revised Atlanta classification system is important before deciding the appropriate treatment strategy because different complications of SAP are treated in different ways, either conservatively by interventional imaging techniques or by surgery. Although no universally accepted treatment algorithm exists, the step-up approach using close monitoring, percutaneous or endoscopic drainage, followed by minimally invasive video-assisted retroperitoneal debridement has been shown to produce superior outcomes to traditional open necrosectomy and may be considered as the reference standard intervention for this disorder. Several recently published studies showed that the step-up approach, compared with open debridement (step-down approach), reduced the rates of major complications and death by minimizing surgical trauma in already critically ill patients with necrotizing pancreatitis. The individual components of the step-up approach may be subject to

improvement. Additional research, preferably randomized trials or prospective collaborative studies, are required to improve current minimally invasive interventional techniques (drainage, endoscopic and laparoscopic) and to define optimal duration and timing of each intervention as part of the step-up approach. The primary principle of intervention for necrotizing pancreatitis is that there is no unique treatment that is optimal for all patients. The best approach is a multidisciplinary one that is adaptable to the individual patient. Therefore, for the management of such complex disease entities, a multidisciplinary team approach is essential, and the final selection of the optimal treatment of SAP will depend on multiple factors, including the expertise available at a given center, specific patient characteristics and risk assessment findings.

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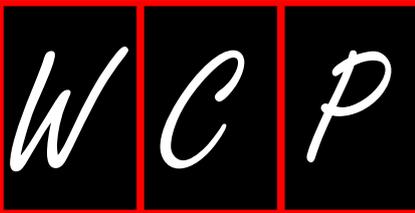
2014 ADVANCES IN PANCREATITIS

Magnetic resonance imaging of pancreatitis: An update

Sriluxayini Manikkavasakar, Mamdoh AlObaidy, Kiran K Busireddy, Miguel Ramalho, Viragi Nilmini, Madhavi Alagiyawanna, Richard C Semelka

Sriluxayini Manikkavasakar, Mamdoh AlObaidy, Kiran K Busireddy, Miguel Ramalho, Viragi Nilmini, Madhavi Alagiyawanna, Richard C Semelka, Department of Radiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7510, United States

Author contributions: Manikkavasakar S, AlObaidy M, Busireddy KK, Ramalho M, Nilmini V, Alagiyawanna M, and Semelka RC contributed equally to this work, including literature review, manuscript writing, manuscript editing, figures collection, writing figures captions, and figures annotations.

Correspondence to: Richard C Semelka, MD, Department of Radiology, University of North Carolina at Chapel Hill, 2001 Old Clinic Bldg., CB 7510, Chapel Hill, NC 27599-7510, United States. richsem@med.unc.edu

Telephone: +1-919-9669676 Fax: +1-919-8437147
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Core tip: Magnetic resonance (MR) imaging is widely used in the diagnosis and staging of pancreatitis, and may represent the best imaging techniques due to its unmatched soft tissue contrast resolution, non-ionizing nature, higher safety profile of intravascular contrast media. This review addresses new trends in clinical pancreatic MR imaging emphasizing its role in imaging all types of acute and chronic pancreatitis, autoimmune pancreatitis, pancreatitis complications, and other important differential diagnoses that mimic pancreatitis.

Abstract

Magnetic resonance (MR) imaging plays an important role in the diagnosis and staging of acute and chronic pancreatitis and may represent the best imaging technique in the setting of pancreatitis due to its unmatched soft tissue contrast resolution as well as non-ionizing nature and higher safety profile of intravascular contrast media, making it particularly valuable in radio-sensitive populations such as pregnant patients, and patients with recurrent pancreatitis requiring multiple follow-up examinations. Additional advantages include the ability to detect early forms of chronic pancreatitis and to better differentiate adenocarcinoma from focal chronic pancreatitis. This review addresses new trends in clinical pancreatic MR imaging emphasizing its role in imaging all types of acute and chronic pancreatitis, pancreatitis complications and other important differential diagnoses that mimic pancreatitis.

INTRODUCTION

Pancreatitis is a major cause for abdominal pain and hospitalization in the United States and worldwide. Incidence of pancreatitis is increasing in the recent decades[1,2]. Imaging plays a central role in the management and complications of pancreatitis[3]. The recent development of new respiratory gating techniques, motion resistant pulse sequences, and additive advantages of magnetic resonance cholangiopancreatography (MRCP) imaging protocols make magnetic resonance imaging (MRI) a very accurate investigation modality for assessing patients with pancreatitis[4,5], particularly acutely ill patients unable to breath hold[6]. This review addresses new trends in clinical pan-

creatic MR imaging emphasizing its role in imaging all types of acute and chronic pancreatitis, pancreatitis complications and other important differential diagnoses that mimic pancreatitis.

MRI and MRCP are the most safe, effective, and noninvasive imaging method for evaluation of the pancreas and ductal system[7]. Technical innovations in MRI such as the use of phased-array coils and parallel imaging allow for improved spatial resolution and faster acquisition times. The use of triggering techniques[8] or motion resistant sequences such as free-breathing three-dimensional gradient-echo with radial data sampling (Radial 3D-GRE), make routine MRI of the pancreas more feasible[6]. MRI has the unique capability of allowing noninvasive evaluation of the pancreatic parenchyma, pancreatic ductal system, peripancreatic soft tissue, and vascular network in a single examination. The concurrent use of Secretin improved the diagnostic yield of MRCP in the evaluation of the pancreatic duct integrity and pancreatic exocrine function in cases of early pancreatitis[9-11].

Normal pancreatic parenchyma has high aqueous protein content that results in high signal intensity on T1-weighted fat-suppressed breath-hold gradient-echo sequences[12] and shows uniform enhancement on the hepatic arterial-dominant phase, also known as late hepatic arterial phase (Figure 1). The standard MR protocol includes a fat-suppressed 3D-GRE T1-weighted pre- and post-gadolinium imaging in the capillary phase (hepatic arterial-dominant phase), portal, and interstitial phase (3-10 min post-Gadolinium)[13]. The main advantages of these sequences are being able to acquire thinner sections (3 mm) and acquire multiplanar imaging. On gadolinium-enhanced images, the pancreas demonstrates a uniform capillary blush on immediate post-Gadolinium images, which renders it markedly higher in signal intensity than liver, neighboring bowel, and adjacent fat[14]. By 1-min post-Gadolinium, the pancreas shows approximately isointense signal to fat on non fat-suppressed T1-weighted GRE, and moderately higher signal than background fat in fat-suppressed SGE or 3D-GE sequences.

Echo-train spin-echo sequences such as T2-weighted half-Fourier acquisition snapshot turbo spin-echo (HASTE) are motion robust sequences that provide a sharp anatomic delineation of the common bile duct (CBD) on coronal plane images and of the main pancreatic duct on transverse plane images. Also, T2-weighted images provide information on the complexity of the fluid within pancreatic pseudocysts, which may reflect the presence of complications such as necrotic debris or infection.

MRI combining T1, T2, early and late post-gadolinium images, MRCP, and MRA generate comprehensive information on the pancreas[13,15].

Three-dimensional MRCP images are acquired in the plane of the pancreatic duct in an oblique coronal projection with the additive advantage of yielding multiplanar maximal projection (MIP) and volume rendering (VR) imaging (Figure 2). It delineates longer segments of the pancreatic duct in continuity[13,16].

PANCREATITIS

Pancreatitis is defined as the inflammation of the pancreas and considered the most common pancreatic disease in children and adults. It can be acute; representing an acute inflammatory process of the pancreas, or chronic; progressing slowly with continued, permanent inflammatory injury to the pancreas.

Acute pancreatitis

Acute pancreatitis is an acute inflammatory process of the pancreas, which may spread to adjacent tissues and organs[17,18]. It can be triggered by several factors, of which alcoholism and choledocholithiasis are responsible for 90% of cases in the United States[17]. The diagnosis of non-complicated acute pancreatitis mainly depends on elevated serum amylase or lipase level of more than three time of its upper limit with characteristic clinical findings[18,19]. In severe forms of the disease, imaging is performed to assess pancreatic parenchyma perfusion, the extent of necrosis as well as the presence and extent of fluid collections and other complications.

The revised Atlanta classification for acute pancreatitis in 2012[20] was aimed to establish international standards of definitions of acute pancreatitis and its complications and to ensure the proper communication between multidisciplinary team working in these patients[20]. According to this classification early imaging will only be required when patient is suspected to have pancreatitis without elevated serum amylase or lipase.

Classification of acute pancreatitis

According to the revised Atlanta classification, acute pancreatitis can be divided into interstitial edematous and necrotizing pancreatitis[20].

Interstitial edematous pancreatitis

It is commonly present as a generalized enlargement of pancreas, or in some cases, as focal swelling due to inflammatory edema of the pancreas and surrounding peripancreatic fat (Figure 3) and relatively homogenous enhancement with contrast (Figure 4). Some peripancreatic fluid may also be present (Figure 5). The clinical symptoms of interstitial edematous pancreatitis usually resolve within first week of onset of disease[21].

Necrotizing pancreatitis

About 5%-10% of cases develop necrosis of pancreatic tissue, peripancreatic fat, or both (Figure 6).

Development of pancreatic and peripancreatic necrosis due to the impaired perfusion in acute pancreatitis takes several days[22,23]; explaining why initial imaging may underestimate disease progression. Initial images may show patchy non-enhancing areas that become more confluent or diffuse with time[22,23] (Figure 7). Non-enhancing areas after 7 d of the disease are diagnosed as paren-
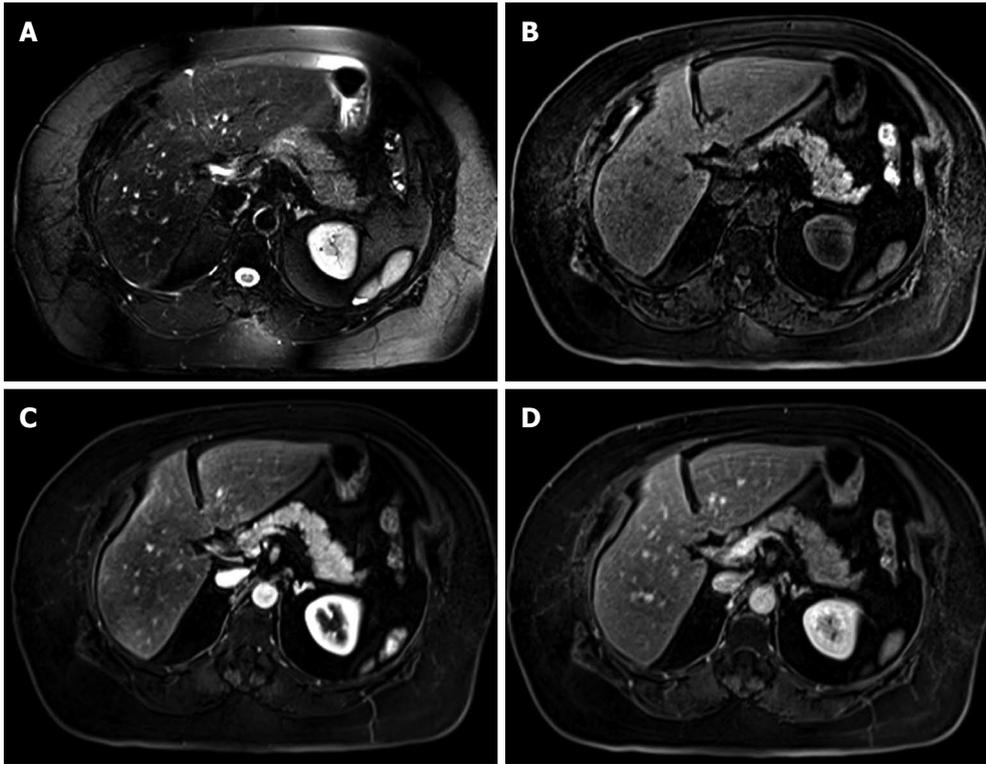
Figure 1 Normal pancreatic appearance on magnetic resonance imaging. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial pre-contrast 3D-GRE T1-weighted image with fat-suppression. Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant (C) and hepatic-venous phases (D). The pancreas demonstrates low T2 signal intensity (A) and high T1 signal intensity on pre-contrast images (B) reflecting high protein content of the exocrine gland. The pancreas demonstrates maximal enhancement on hepatic arterial-dominant phase (C); which fades on subsequent phases; reflecting a normal capillary blush.
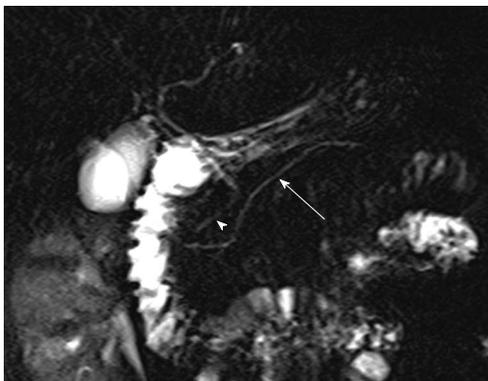
Figure 2 Normal magnetic resonance cholangiopancreatogram. Coronal oblique thick-slab magnetic resonance cholangiopancreatogram (MRCP) image. There is normal course and normal uniform diameter of the pancreatic (arrow) and extra-hepatic biliary ducts (arrowhead). This sequence requires less than 1 s to acquire and is very sensitive for detecting stones.

chymal necrosis[24]. Normal enhancement of pancreatic parenchyma may be seen in peripancreatic necrosis, and is associated with increased rate of morbidity than in patients with interstitial edematous pancreatitis[21,22,25]. Pancreatic and peripancreatic necrosis can be infected, liquefied, remain solid, sterile or in some cases persist or disappear with time.

Complications of acute pancreatitis
According to the revised Atlanta classification[20] for pancre-

atitis, the following are the complications noted on imaging.

Acute peripancreatic fluid collections: They occur early in the course of the disease[20,26]. They present between the facial planes surrounding the pancreas and do not have clear wall around them. They can be single or multiple, but their contents are typically homogenous and sterile. Patients with Acute peripancreatic fluid collection (APFC) are asymptomatic and treatment is unnecessary. Most of these collections resolve on their own[26,27]. If they do not resolve within a month, they may become pancreatic pseudocysts (Figure 8).

Pancreatic pseudocysts: Cystic lesions with homogenous internal fluid content, but without any solid materials, demarcated by a clear wall and located in the pancreatic or peripancreatic regions are called pseudocysts (Figures 9 and 10). High amylase levels are seen in aspirated fluid from these cysts[20].

Pancreatic pseudocysts develop due to the leakage of pancreatic juice from the ruptured main pancreatic duct or its side branches of more than 4 wk, with no obvious pancreatic necrosis. T2-weighted images confirm the absence of solid content in the collection. Pseudocysts may also arise in the setting of acute necrotizing pancreatitis as a result of a "disconnected duct syndrome" where proximal pancreatic parenchymal necrosis isolates a still viable distal pancreatic parenchymal remnant[28].

Pseudocysts may be evident many weeks following
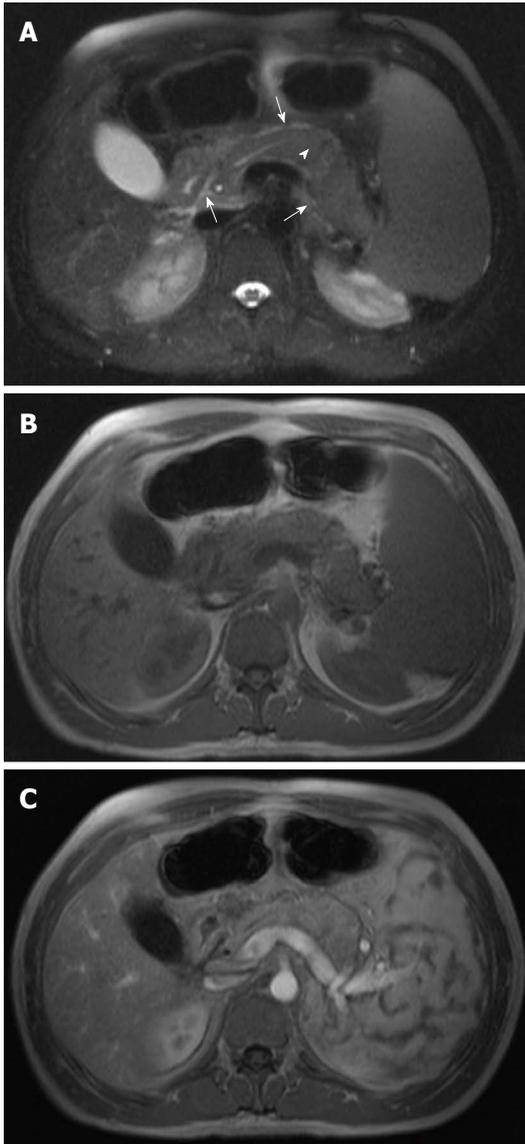
Figure 3 Mild acute interstitial edematous pancreatitis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image; B: Axial pre-contrast 3D-GRE T1-weighted images with fat-suppression; C: Axial post-Gadolinium 3D-GRE T1-weighted images with fat-suppression during the hepatic arterial-dominant phases. There is mild lace-like increased T2 signal involving the pancreatic parenchyma, associated with effacement of the distal pancreatic duct (arrowhead), due to surrounding edema, and minimal amount of peripancreatic fluid around (arrows) the head and body (A). The pancreas demonstrates mildly enlarged distal body and tail (A-C); with diffuse minimally decreased T1 signal intensity (B); and mild heterogeneous enhancement of the distal body and tail on the hepatic arterial-dominant phase (C) in keeping with diffuse edematous pancreatitis.

Figure 4 Gallstone acute interstitial edematous pancreatitis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial in-phase T1-weighted image; C: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase. There is mild diffuse lace-like increased T2 signal involving the pancreatic parenchyma (arrowheads), associated with a small amount of peripancreatic fluid (arrows) (A). The pancreas demonstrates diffuse minimal decreased T1 signal intensity with peripancreatic stranding (B); in addition to minimally reduced homogenous enhancement (C) in keeping in with diffuse edematous pancreatitis. There are also innumerable gallstones (asterisk) (A).

operative necrosectomy secondary to localized leakage of the disconnected duct into the necrosectomy cavity.

Acute necrotic collections: Well-defined collections of variable amount of necrotic materials and fluid within a month of disease occurrence are referred to as acute necrotic collections (ANCs) (Figure 11). They can be single or multiple, and are sometimes multi-loculated. The main differences between ANCs and APFCs are that ANCs contain necrotic materials, and also develop from necrotizing pancreatitis, sometimes associated with pancreatic

ducts disruption, and occasionally they can get infected[20]. Because of CT's low contrast resolution, MRI may be indicated in the early course of the disease to differentiate ANCs from APFCs. With time, parenchymal necrosis becomes more obvious, which aids in the distinction of ANCs from APFCs. MRI is more sensitive in depicting solid tissue within ANCs.

Walled-off necrosis: Well-defined necrotic tissues surrounded by enhancing viable inflammatory walls are referred to as walled-off necrosis (WONs). They can be confined to the pancreatic parenchyma, involve the
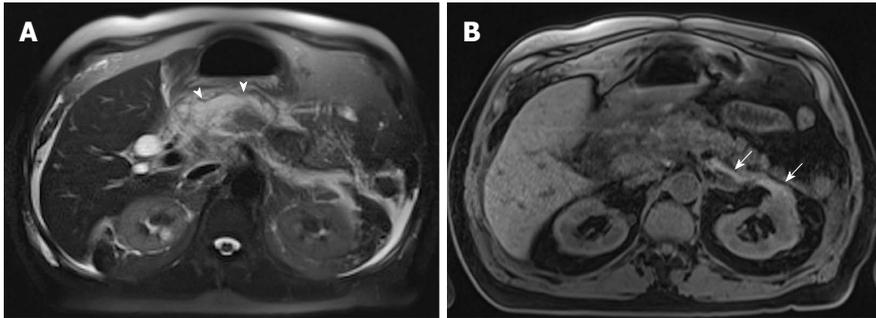
Figure 5 Acute interstitial edematous pancreatitis with acute peripancreatic fluid collection. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial 3D-GRE T1-weighted image with fat-suppression. The pancreas shows mild lace-like increased T2 signal involving the pancreatic parenchyma (A), with minimally decreased T1 signal intensity and mild peripancreatic stranding (B), associated with peripancreatic fluid collections (arrowheads, A) and small proteinaceous fluid at the left anterior para- and peri-renal spaces (arrows); both associated with imperceptible wall in keeping with acute interstitial edematous pancreatitis and acute peripancreatic fluid collection. Minimal ascites is also seen (A).
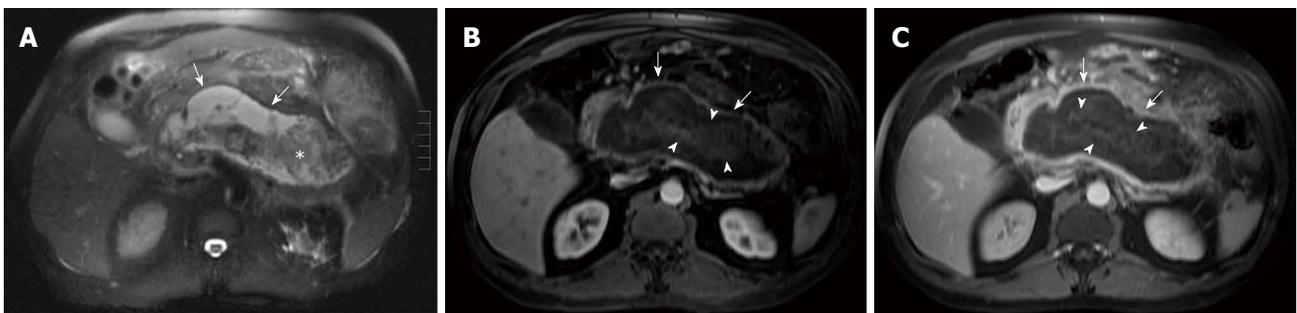
Figure 6 Pancreatic and peripancreatic necrosis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression. Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the B hepatic arterial-dominant and C hepatic-venous phases. The pancreas shows very heterogeneous increased T2 signal (asterisk) (A) and no appreciable enhancement on the post-Gadolinium images (arrowheads) (B, C); associated with a large peripancreatic fluid collection (arrow) (A-C) and a thick enhancing rim on delayed images (C) in keeping with pancreatic and peripancreatic necrosis.
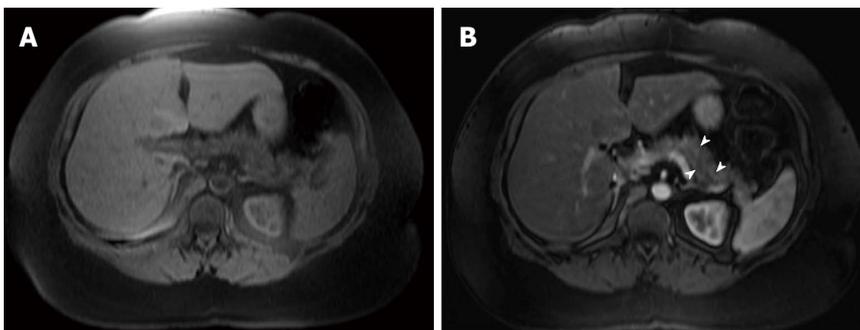
Figure 7 Necrotizing pancreatitis. A: Axial T1-weighted fast low-angle shot (FLASH) image with fat-suppression; B: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase. The pancreas shows diffuse decreased T1 signal intensity (A) with patchy areas of minimal enhancement (arrowheads) (B) in keeping with necrotizing pancreatitis.

peripancreatic tissue (Figure 12), or even at times be away from the pancreas. The whole process may take about a month after the onset of necrotizing pancreatitis. WONs can be sterile or infected as well as solitary or multiple[20]. Contrast-enhanced CT may misdiagnose WONs as pseudocysts. MRI may be useful to differentiate solid from liquid contents within the lesion. Identification of ductal disruptions is necessary for further management.

Infected pancreatic necrosis: The diagnosis of infected pancreatic necrosis is suspected in the presence of extra-

luminal gas or gas-fluid level in the areas of pancreatic or peripancreatic necrosis on imaging[20] (Figure 13). The amount of gas-fluid level depends on the stage of the disease. Confirmation of diagnosis is made by microscopy and culture of fine needle aspiration[29,30]. Infected necrosis is associated with high morbidity and mortality and requires drainage and antibiotic therapy.

Disconnected pancreatic duct syndrome: It is characterized by disruption of the main pancreatic duct with loss of continuity between the pancreatic duct and the
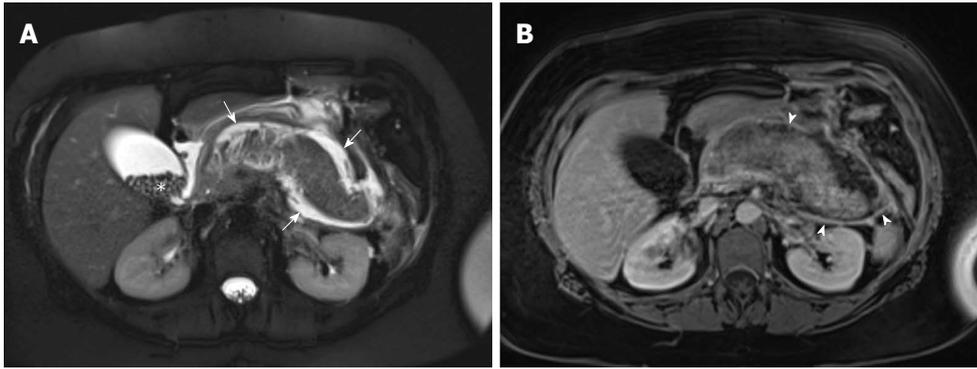
**Figure 8 Gallstone acute edematous pancreatitis with acute peripancreatic fluid collection.** A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression. There is diffuse lace-like increased T2 signal involving the pancreatic parenchyma (A), minimally reduced enhancement post-Gadolinium, peripancreatic stranding, and thick rim of enhancement (arrowheads) surrounding the pancreas (B), associated with peripancreatic fluid collection (arrows) in keeping with diffuse edematous pancreatitis and peripancreatic acute peripancreatic fluid collection. There are also innumerable gallstones (asterisk) (A).
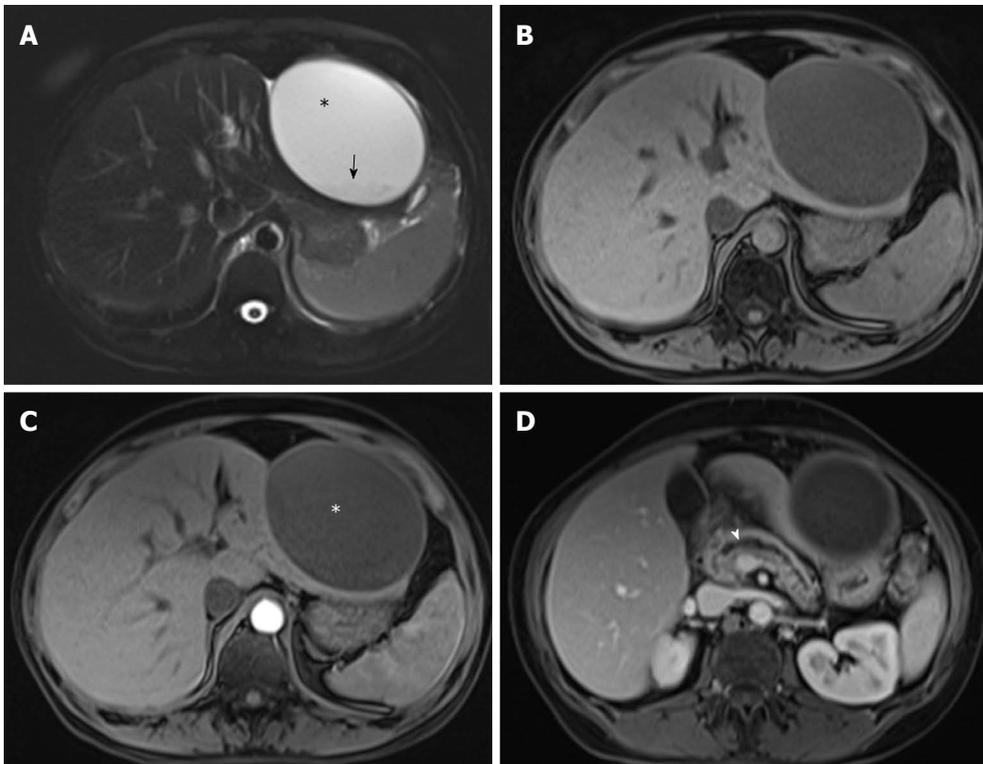
Figure 9 Large pancreatic pseudocyst. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial pre-contrast 3D-GRE T1-weighted image with fat-suppression. Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during (C) the true late hepatic arterial and (D) hepatic-venous phases. There is a large oval shaped thin walled cyst (asterisks) with homogenously increased T2 (A) and decreased T1 signal intensities (B) at the left subdiaphragmatic region, with dependent non-enhancing (C) layering material (arrows) (A, B). The cyst demonstrates mild wall enhancement (C, D) in keeping with a large pancreatic pseudocyst. There is also diffuse pancreatic parenchyma thinning, irregular diffuse main pancreatic ducal dilatation (arrowhead), and pancreatic side-branches ductal prominence in keeping with chronic pancreatitis (D).

gastrointestinal tract caused by ductal necrosis after severe acute necrotizing pancreatitis treated by percutaneous drainage or necrosectomy[20,28].

Diagnosis of disconnected pancreatic duct syndrome is important in the determination of the optimal approach (surgical, endoscopic, and percutaneous) for patients with organizing pancreatic necrosis or fluid collections[31]. The treatment of the disconnected pancreatic duct is surgical and requires either internal drainage or distal pancreatic resection for complete resolution. The exact incidence of this syndrome remains unknown; however, pancreatic duct disruption has been observed in as many as 50% of patients after an episode of severe acute necrotizing pancreatitis[9,31].

The morbidity associated with ERCP in the setting of recent acute pancreatitis is high, and the procedure is
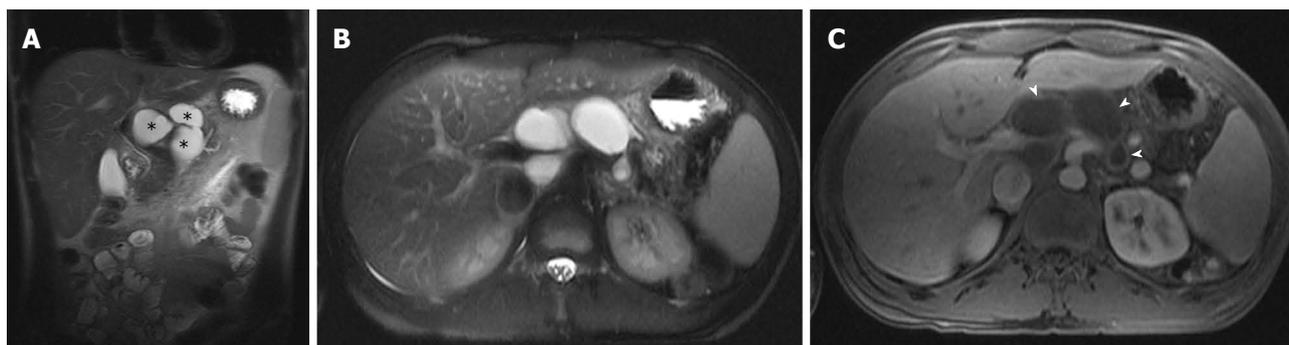
Figure 10 Multiple intrapancreatic pseudocysts. Coronal (A) and axial (B) single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; C: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase. There are multiple thin walled cysts, some of which show multi-loculation (asterisks) (A) within the pancreatic parenchyma extending into the lesser sac and porta hepatis (A-C) and demonstrate mild uniform wall enhancement (arrowheads) (C) in keeping with multiple pseudocysts.
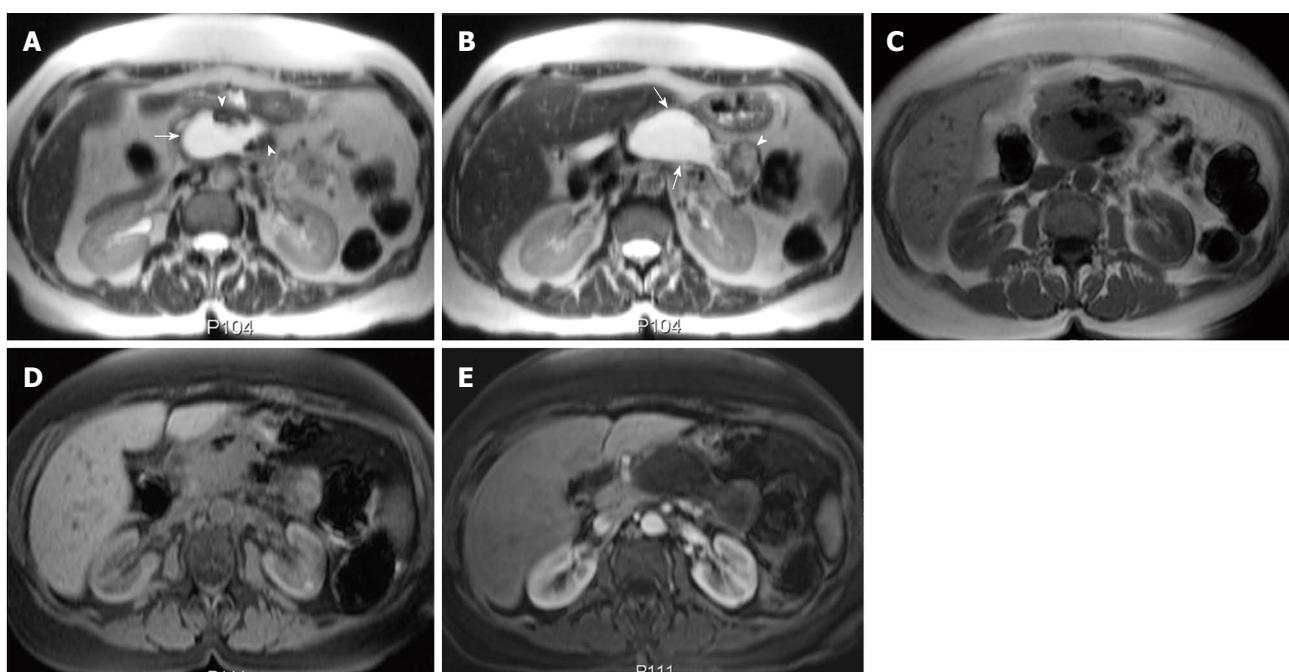
Figure 11 Acute necrotic collection. A, B: Axial single-shot turbo spin-echo T2-weighted (HASTE) image; C: Axial fast low-angle shot (FLASH) in-phase T1-weighted image; D: Axial FLASH in-phase T1-weighted image with fat-suppression; E: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase. There is a well-defined fluid collection (arrows) replacing a great portion of the pancreatic parenchyma and extending into the peripancreatic tissue, associated with few internal areas of decreased T2 signal intensity (arrowheads) (A); which do not show any appreciable enhancement (B, C) in keeping with acute necrotic collection.

often technically challenging because of ongoing edema that involves the duodenum or complete disruption of the main pancreatic duct. MRCP is a non-invasive way of assessing the disconnected pancreatic duct syndrome (DPDS), where discrete intra pancreatic fluid collection along the expected course of the main pancreatic duct, with viable upstream pancreatic parenchyma, is suggestive of the diagnosis of the disconnected pancreatic duct syndrome[9].

ERCP findings of ductal obstruction at the level of this fluid collection, with or without extravasation of contrast material, help in confirming this diagnosis. Although ERCP is still considered the reference standard for the evaluation of disconnected pancreatic duct syndrome,

secretin-enhanced MRCP can be useful in determining whether the main pancreatic duct is disrupted or disconnected in patients with necrotizing pancreatitis[9,11,32-34] (Figure 13).

Other complication: Other local complications of acute pancreatitis include gastric outlet dysfunction, splenic or portal veins thrombosis, and large bowel necrosis. Clinical features of local complications includes recurrent abdominal pain, secondary increase in serum amylase, impaired renal and liver function, fever, and, leukocytosis.

MRI and MRCP in acute pancreatitis
A variety of pulse sequences are routinely used in pan-
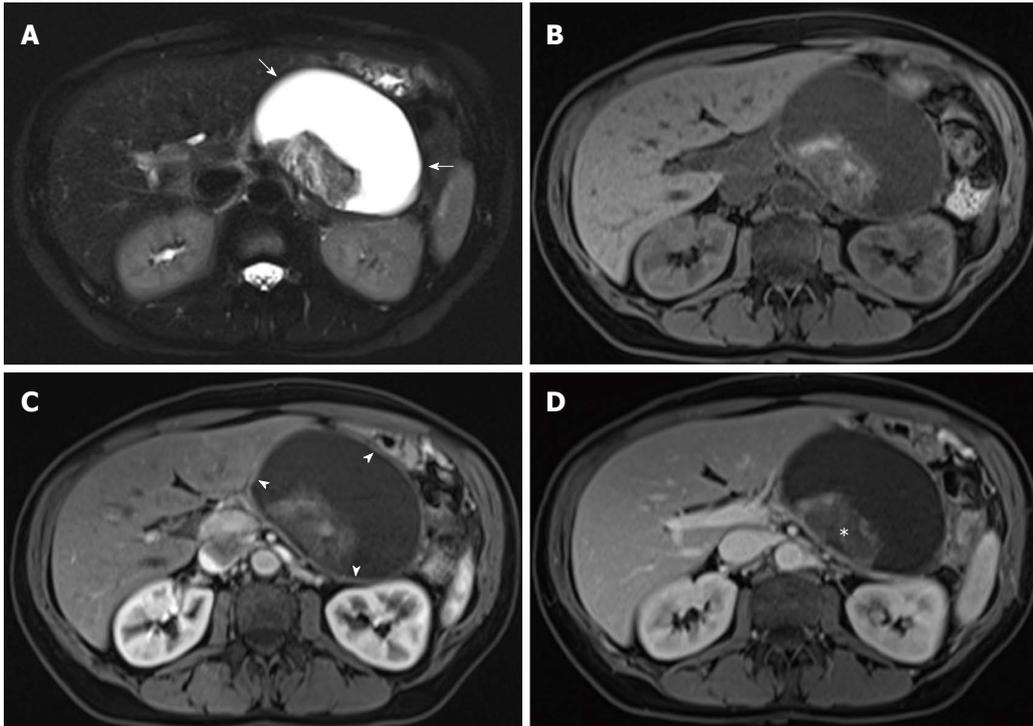
Figure 12 Necrotizing pancreatitis with peripancreatic walled-off necrosis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image. Axial (B) pre- and post-Gadolinium 3D-GRE T1-weighted images with fat-suppression during the hepatic arterial-dominant (C) and hepatic-venous phases (D). There is a well-defined fluid collection (arrows) at the region of the pancreatic body/tail (A, B); which demonstrates a uniform mildly enhancing thickened rim (arrowheads) (C,D) and contains a dependent non-enhancing debris (asterisk) (C, D) in keeping with necrotizing pancreatitis and walled-off necrosis.
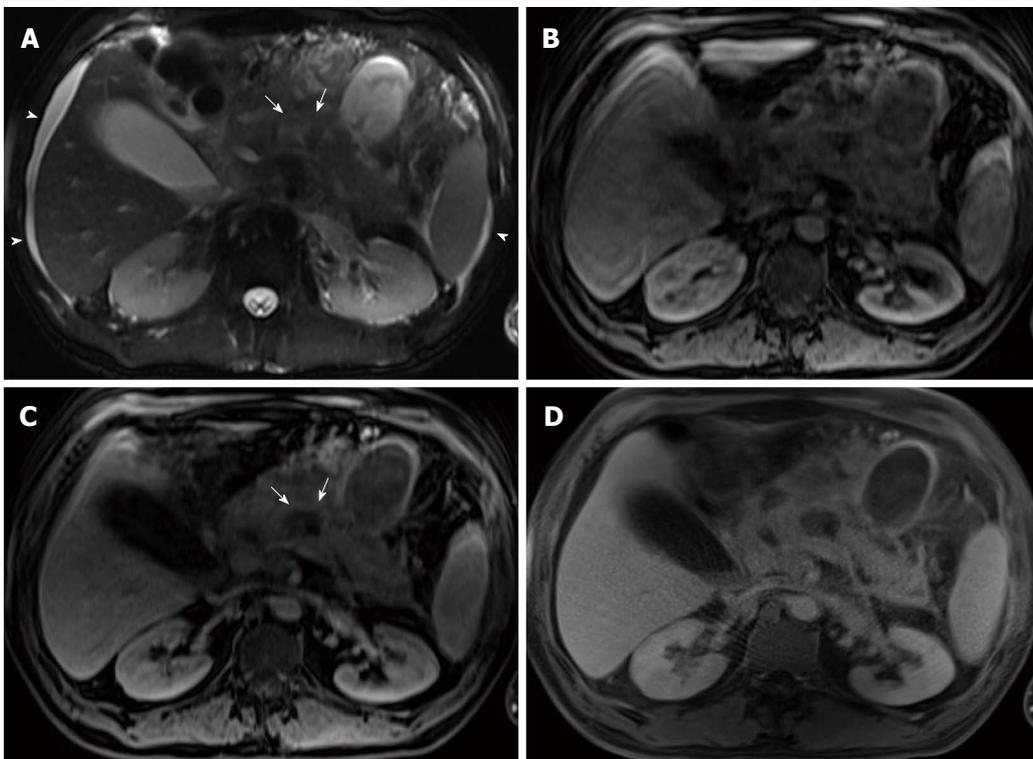
Figure 13 Infected peripancreatic fluid and interrupted duct syndrome in a patient with acute pancreatitis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression. Axial pre- (B) and post- (C) Gadolinium 3D-GRE T1-weighted images with fat-suppression during the hepatic arterial-dominant phase; D: Axial post-Gadolinium radial 3D-GRE T1-weighted images with fat-suppression during the interstitial phase. There is a large irregular multiloculated (partially visualized) peripancreatic collection. It demonstrates decreased T2 (A) and T1 (B) signal with a thick rim of increased T2 signal (arrows) (A) that shows enhancement on delayed images (arrows, C, D) in keeping with infected necrotic collection (proven by fine needle aspiration). There is also a well-defined fluid collection in the lesser sac (asterisk) (A) with an enhancing thin wall (D) and minimal ascites (arrowheads, A). Of note is the motion robustness of the radial 3D-GRE (D) compared to convention 3D-GRE (C) sequence, facilitating imaging of sick uncooperative patients.
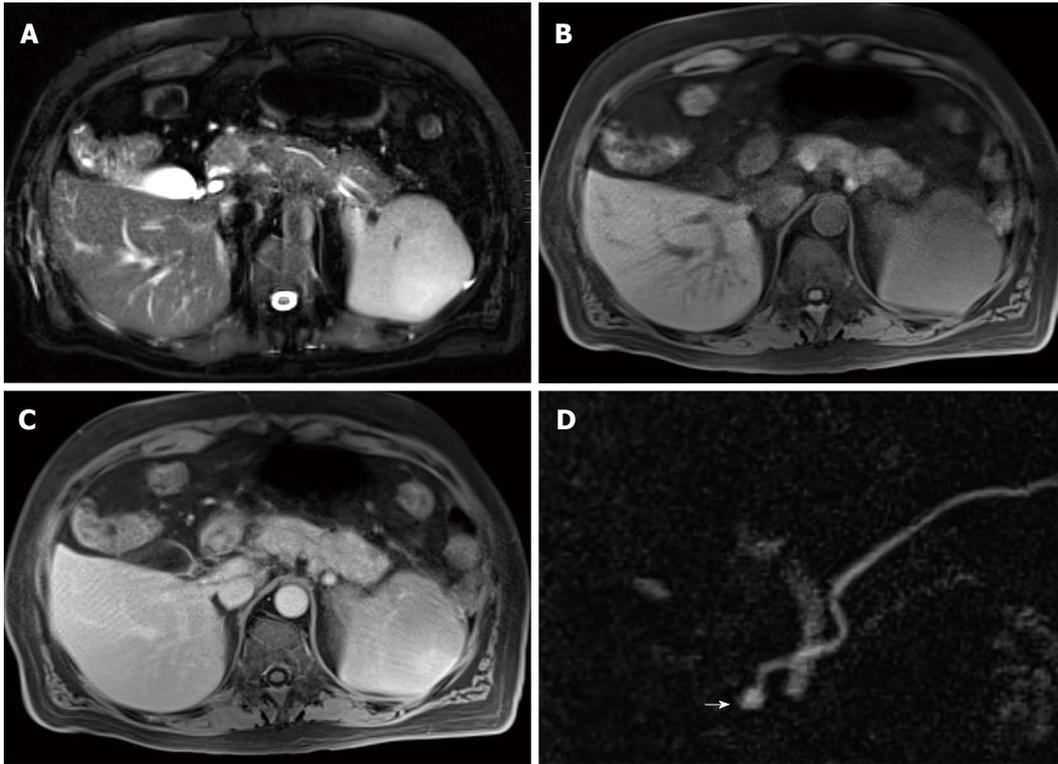
Figure 14 Mild pancreatitis complicated by pancreatic divisum and santorinicele. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression. Axial pre- (B) and post- (C) Gadolinium radial 3D-GRE T1-weighted image with fat-suppression during the hepatic-venous phase; D: Coronal oblique thick-slab magnetic resonance cholangiopancreatogram image. There is mild lace-like increased T2 signal involving the pancreatic parenchyma, associated with a minimal amount of peripancreatic fluid (A) and pachy pancreatic enhancement on post-Gadolinium images (B, C) in keeping with mild acute pancreatitis. There is also abnormal course and insertion of the main pancreatic duct into the minor papilla (D) terminating in a small cystic structure (arrow) in keeping with pancreatic divisum and a small Santorinicele (D).

creatic imaging including T1- and T2-weighted sequences with or without fat-suppression and 3D-GRE T1-weighted pre- and post-gadolinium administration. MRCP is routinely added to the standard imaging protocol to assess ductal obstruction, dilation, anatomical variation (Figure 14), or complication such as disconnected pancreatic duct syndrome. In selected cases, secretin-enhanced MRCP further increases the diagnostic value of MRCP[9,11,32-34]. T2-weighted fat-suppressed images are extremely sensitive in detecting subtle, early pancreatitis in patients who have negative CT scan findings[19]. It has been reported that MR severity index (MRSI) assessed by using 0.5 Tesla (T) MR systems without contrast significantly correlated with CT severity index (CTSI), Ranson score, C-reactive protein levels, appearance of systemic complications, duration of hospitalization, and clinical outcome[35,36].

CHRONIC PANCREATITIS

Chronic pancreatitis is defined as continuing inflammatory destruction of pancreatic tissue that results in irreversible structural changes of pancreas including parenchymal tissue and ductal system. The incidence of chronic pancreatitis is 5-12 per 100000 persons per year; accounting for more than 120000 outpatient visits and 50000 hospitalizations annually[37]. Alcohol consumption is a major cause in adults (80%) in developed countries,

whereas malnutrition is the most common cause worldwide[38]. Other causes of chronic pancreatitis includes genetic mutations, pancreatic ductal obstruction or anatomical variation, hypercalcemia, nutritional factors, hyperparathyroidism, and hyperlipidemia.

Diagnosis is made by clinical history, testing of pancreatic exocrine function, and imaging[39]. MRI is more reliable in diagnosing chronic pancreatitis than CT or ultrasonography[40]. MRI will identify parenchymal atrophy, duct dilation, and pancreatic ductal and parenchymal changes after hormonal stimulation[40,41]. Chronic pancreatitis damages the acinar cells, main pancreatic duct, and side branches. Microscopic finding includes sclerosis and fibrosis of pancreatic duct and side branches, acinar cell atrophy and ductal dilatation stricture or stenosis. Eosinophilic protein plugs or intraductal calcification may be seen with the ducts. All these changes will lead to focal, segmental or generalized atrophy or loss of pancreatic tissue, main and/or side pancreatic ducts stenosis, stricture or dilatation[40-44].

Early chronic pancreatitis

Parenchymal changes might be preceded by ductal changes in chronic pancreatitis[45]; this makes MRCP alone more advantageous in suspected early chronic pancreatitis. MRI detects not only morphological characteristics, but also early fibrotic changes. Fibrosis is shown by di-
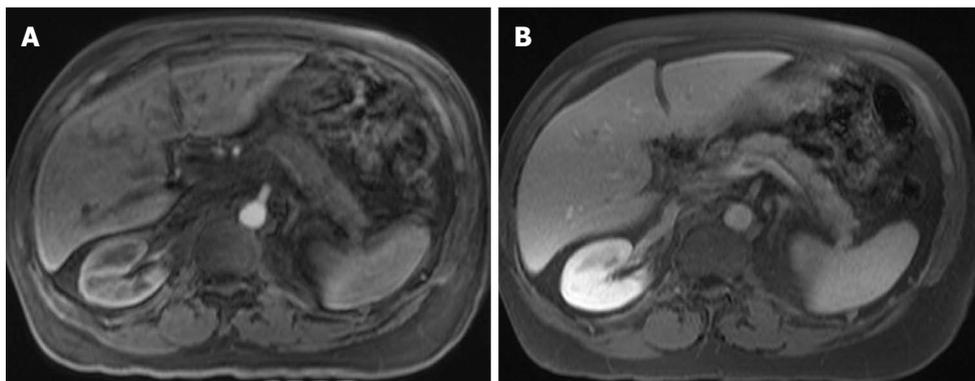
Figure 15 Chronic pancreatitis. A: Axial post-Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase; B: Axial post-Gadolinium FLASH in-phase T1-weighted image with fat-suppression during the hepatic-venous phases. The pancreatic body and tail show poor early contrast enhancement (A) and mild enhancement on the delayed images related to fibrosis (B) in keeping with chronic pancreatitis.
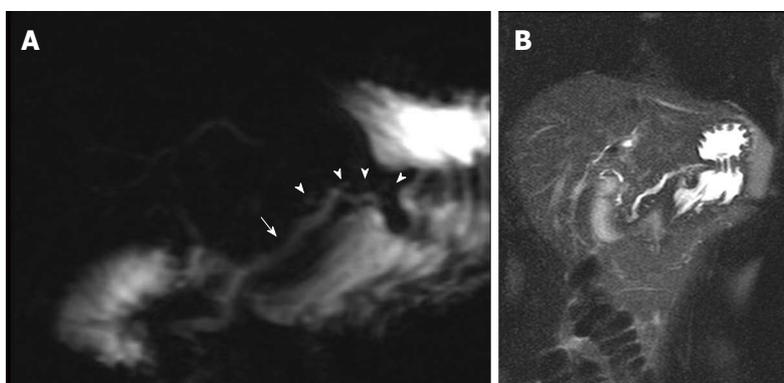
Figure 16 Mild chronic pancreatitis. A: Coronal oblique thick-slab magnetic cholangiopancreatogram image; B: Coronal single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression. There is uniform dilatation of the pancreatic duct (arrow) and prominence of the pancreatic duct side-branches (arrowheads), without significant diffuse thinning of the pancreatic parenchyma (A, B) in keeping with mild chronic pancreatitis.

minished signal intensity on fat-suppressed T1-weighted images and diminished parenchymal enhancement on immediate post-Gadolinium images[46] (Figure 15). Low signal intensity on fat-suppressed T1-weighted images reflects loss of the aqueous protein within the pancreatic acini. Diminished pancreatic parenchymal enhancement on the capillary phase images reflects disruption of the normal capillary bed and increased chronic inflammation and fibrosis. MRCP findings in early chronic pancreatitis often demonstrate normal main pancreatic duct with dilated and irregular side duct branches (Figure 16). The limiting factor is the underestimation of ductal size. Some investigators reported that patients with abnormal MR imaging findings but normal MRCP might benefit from dynamic secretin-MRCP (S-MRCP), which may reveal ductal abnormalities due to improved visualization otherwise not detected on MRCP alone[45]. Secretin-MRCP has been reported to show ductal changes, like dilatations and strictures in early chronic pancreatitis.

Late chronic pancreatitis
All patients with late or advanced chronic pancreatitis show diminished signal intensity of the pancreas on T1-weighted fat-suppressed images, an abnormally low per-

centage of contrast enhancement on arterial phase images, and progressive parenchymal enhancement on the 5-min delayed post-Gadolinium images; reflecting the pattern of enhancement of fibrous tissue[47,48]. MRCP in advanced phase demonstrates dilatation of the main pancreatic duct with ectasia of the side branches; giving chain of lakes appearance (Figure 17) manifested as pancreatic ductal strictures, irregularities and intra ductal calculi, appearing as hypointense filling defects. Other findings include intra ductal calcifications, which are the most specific finding, seen in nearly half of the patients with chronic pancreatitis and parenchymal atrophy (Figure 18), though it is neither specific nor sensitive as it is seen normally with aging. Intraductal or parenchymal calcifications are usually seen with alcohol related chronic pancreatitis but not on chronic pancreatitis resultant from other causes.

Pancreatic inflammatory mass vs pancreatic cancer
Chronic inflammatory process in chronic pancreatitis can produce a focal mass lesion, especially in the head of the pancreas that can mimic more sinister pathologies *i.e.*, pancreatic adenocarcinoma[49] (Figure 19).

Both chronic pancreatitis and adenocarcinoma show similar imaging characteristics on MRI due to abundant

Figure 17 Chronic pancreatitis. Coronal oblique maximal intensity projection image of a 3D magnetic cholangiopancreatogram image. There is mild pancreatic main ductal (arrow) and side branches (arrowheads) dilatation in keeping with chronic pancreatitis.

fibrosis and ductal obstruction; therefore, making the differentiation between these two entities very difficult. Both are generally seen as mildly hypointense on T1-weighted images and heterogeneously mildly hyperintense signal on T2-weighted images. Other possible similar findings in both conditions include common bile duct and main pancreatic duct dilation (double duct sign), stricture of ducts, peripancreatic fat stranding due to infiltration, vascular encasement of superior mesenteric artery, and splenic vein thrombosis[50,51]. T1- and T2-weighted images alone cannot differentiate both conditions. The following supporting feature may favor inflammatory origin: part of the duct seen through the mass is not dilated and shows smooth tapering (duct penetrating sign)[52]. Features favoring carcinoma include abrupt cut off of pancreatic duct just proximal to the ampulla, pancreatic atrophy distal to the mass, and increased duct caliber-to-gland ratio (ductal diameter is out of proportion to pancreatic parenchymal thickness). Pancreatic adenocarcinoma can develop in patients with chronic pancreatitis, which further limits the diagnostic capability of imaging necessitate histological confirmation[51,53]. Excessive fibrosis seen in both conditions explains the similarities on imaging. Because of the similarities between these conditions on clinical and imaging evaluations, histology is mandatory for final diagnosis[50].

Complications of chronic pancreatitis

The most common non-neoplastic complications of chronic pancreatitis include pseudocysts, pseudoaneurysms (due to erosion of the arterial wall), splenic vein thrombosis with subsequent development of collaterals, biliary obstruction (due to pseudocysts), and gastrointestinal complications; such as gastric outlet obstruction or bowel ischemia[19,54]. These complications are well depicted with MRI. MRI with MRCP may be superior to CT in detecting specific complications like pseudocysts, fistula formation, and distal common biliary dilatation. Vascular complications associated with higher morbidity and mortality.
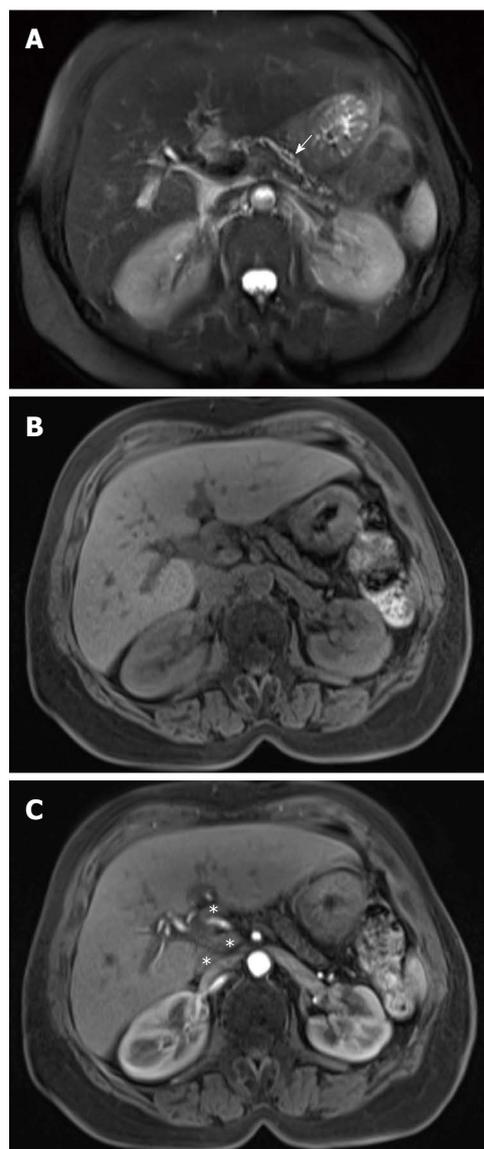
Figure 18 Chronic pancreatitis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image. Axial pre- (B) and post- (C) Gadolinium 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial-dominant phase. There is evidence of diffuse atrophy of the pancreatic parenchyma with mild uniform pancreatic ductal dilatation (arrow) and pancreatic side-branches prominence (A), associated with diminished T1 signal intensity (B) and minimal arterial enhancement (C) in keeping with chronic pancreatitis. There are also few prominent lymph nodes in the peripancreatic and porta hepatis lymph nodes (asterisks) (A-C).

MRI and MRCP in chronic pancreatitis

MRI, combined with MRCP, is an excellent modality to assess patients with clinically suspected chronic pancreatitis[40], MRI evaluates ductal alterations and obstructive causes of chronic pancreatitis like ductal calculi. MRI also assesses signal changes of gland, glandular volume depletion or atrophy, and pancreatic perfusion on contrast-enhanced images. Volume depletion manifests as reduction of anterior posterior diameter of pancreas in the entire gland or in specific segments. Pancreatic fibrosis causes reduction of pancreatic signal intensity on MRI. The normal pancreas is high in signal intensity on
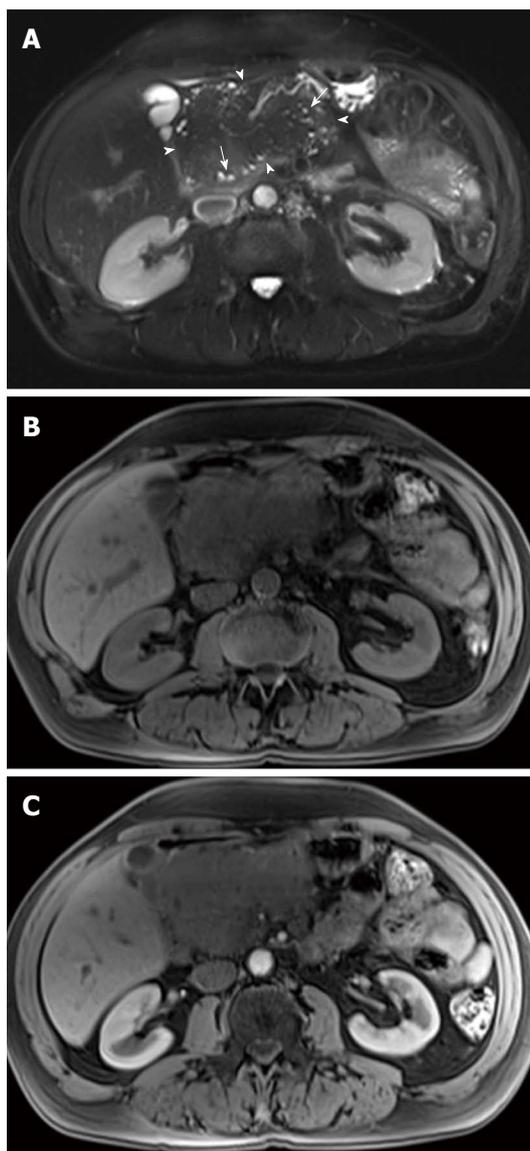
Figure 19 Chronic inflammatory mass simulating pancreatic adenocarcinoma. A: Axial T2-weighted image with fat-suppression; B: Pre-contrast 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial dominant phase; C: Post-contrast 3D-GRE T1-weighted image with fat-suppression during the hepatic arterial dominant phase. There is a complex lobulated pancreatic head mass (arrowheads) with multiple foci of increased T2 signal throughout (arrows) (A), associated with decreased pancreatic parenchymal T1 signal (B), proximal pancreatic ductal dilation (not shown), and diminished enhancement on post-Gadolinium images (C) in keeping the diagnosis of focal chronic pancreatitis.

Table 1 Evaluation criteria for magnetic resonance imaging and magnetic resonance cholangiopancreatography according to Cambridge classification

Cambridge 0	None
Cambridge 1	Not identifiable MRCP abnormalities
Cambridge 2	Two or more of the following abnormalities:
	Pancreatic duct 2 to 4 mm in the body of the pancreas
	Mild pancreatic enlargement
	Heterogeneous parenchymal structure
	Small cysts (< 10 mm)
	Duct irregularities
	More than 3 abnormal side branches
Cambridge 3	All the abnormalities listed in 2, above, along with abnormal main duct (> 4 mm)
Cambridge 4	One of the abnormalities listed in 2 or 3, above, and one or more of the following:
	Cystic structures > 10 mm
	Parenchymal calcifications
	Intraductal filling defects (calcium stones)
	Duct obstruction (stricture)
	Major irregularity of duct

MRCP: Magnetic resonance cholangiopancreatography.

In cases of chronic pancreatitis, a plateau-like pattern is observed. Periductal fibrosis causes dilated main and side pancreatic ducts due to traction[40,41,43,55,56]. Several classification systems are used to define and characterize the severity of chronic pancreatitis. The Cambridge classification, which is the most commonly used grading system for chronic pancreatitis, was established in 1984 for ERCP[57]. This system classifies pancreatograms into normal or equivocal, mild, moderate, and severe changes of chronic pancreatitis on the basis of the main pancreatic duct dilatation, side-branch dilatation, and additional features (Table 1). The new MR techniques with addition of secretin make it possible to use Cambridge classification for MRCP as well by increasing ductal filling to improve the depiction of the pancreatic ductal system[9,11,56,58-60].

Secretin-enhanced MRCP

Secretin-enhanced MRCP is an imaging modality that not only helps identify the characteristic ductal changes of acute or chronic pancreatitis but also provides an estimate of pancreatic excretory volume. It is important to remember that the presence of normal duodenal filling does not exclude impairment of pancreatic exocrine function, which is measured by determining the fluid bicarbonate level[11,61]. Secretin is a polypeptide hormone made by 27 amino acids, secreted by the duodenum. After meals, acid secretion increases in duodenum stimulating the secretion of secretin. Secretin stimulates the pancreas; which secretes bicarbonate rich fluid and increases the tone of sphincter of Oddi. The main pancreatic duct distends by the accumulation of the pancreatic juice due to the effect of secretin. The distention will be maximal in about four to ten minutes. Ductal anatomy can be clearly studied after secretin stimulation. Other advantages of secretin are ease of administration and safety of use with low incidence of major side effects[9,11,61-63]. Excretory function of the pancreas is graded in secretin-

fat-suppressed T1-weighted images due to the presence of aqueous protein in the acini. Normally, the pancreas shows a uniform capillary blush on the late arterial phase images and fades on the subsequent hepatic venous and delayed images. In patients with chronic pancreatitis, loss of aqueous protein in the acini causes reduction of signal on fat-suppressed T1-weighted images. The pancreas shows progressive enhancement that peaks on the portal venous or interstitial phase. This finding is the result of pancreatic fibrosis that causes impairment of capillary blood flow to the gland. A time-related perfusion model can assess pattern of enhancement of the pancreas, where normal pancreas shows high upslope in contrast.

enhanced MRCP according to the duodenal anatomic imaging findings: grade 1, when pancreatic fluid is confined to the duodenal bulb; grade 2, when fluid is seen as far as the second part of the duodenum; and grade 3, when duodenal filling reaches the third part of the duodenum. Diminished estimated pancreatic exocrine function is suspected in the absence of duodenal fluid accumulation, or with grade 1 duodenal filling[11,61]. This grading does not differentiate between early and established pancreatitis. To adequately assess the exocrine response to secretin, patients should be fasting for at least 4 h before the MR imaging examination. It is recommend that the administration of a negative oral contrast agent to remove high signal intensity from the fluid within the stomach and duodenum on MRCP images. Ferumoxsil suspension (300 mL) can be used as an oral contrast agent. Other oral contrast agent is diluted gadolinium-DTPA (5 mL gadolinium DTPA diluted with 75 mL of distilled water). Oral contrast agents shorten the T2 time and act like a negative T2 agent[55,64-67]. If a commercial product is not available, pineapple juice or blueberry juice can be used as alternative negative MR contrast material. Oral contrast agent should be given 30 min before the procedure to counteract signals from preexisting duodenal secretions. Secretin bolus injection can cause abdominal cramps; to avoid this, slow intravenous infusion over a minute is indicated. Following negative oral contrast administration and intravenous infusion of 0.2 μg secretin, serial coronal single-shot fast spin echo images are obtained every 30 s for 15 min from the time of injection[44,45,67,68].

Diffusion weighted imaging and chronic pancreatitis

Diffusion weighted imaging (DWI) sequence may be used to assess pancreatic parenchymal atrophy in patients with chronic pancreatitis, where atrophy and ductal pathology are subtle, and has shown results comparable to MRI and MRCP[48,69]. DWI assesses the random microscopic motion of water protons to obtain the apparent diffusion coefficient (ADC). This is reflected by reduction in ADC values in case of chronic pancreatitis as loss of exocrine functional tissue of pancreas increases the amount of fibrosis, leading to reduction in water diffusibility[40,60]. Furthermore, when DWI is used in combination with secretin stimulation, the diffusion coefficients have either delayed or lower peak values in chronic pancreatitis, indicating reduced exocrine function[48,70,71].

Special types of chronic pancreatitis

Autoimmune pancreatitis: Continuous inflammatory process of the pancreas due to an autoimmune pathogenesis leading to chronic pancreatitis called autoimmune pancreatitis. Other autoimmune diseases associated with autoimmune pancreatitis (AIP) include ulcerative colitis, primary sclerosing cholangitis, primary biliary cirrhosis, retroperitoneal fibrosis, Sjogren's syndrome, and systemic lupus erythematosus[72]. It is accounts for 2%-6% of chronic pancreatitis[73,74]. It is characterized, clinically, by obstructive jaundice (with or without pancreatic mass);

histologically, by a lymphoplasmacytic infiltrate and fibrosis; and, therapeutically, by a dramatic response to steroids[75]. Early diagnosis of AIP is critical as it often responds well to steroid therapy; thus avoiding complications.

There are three types based on morphological patterns: diffuse, focal, and multifocal. Diffuse disease is the most common type. MRI commonly shows a swollen, sausage-like pancreas (Figure 20) with poorly demonstrated borders, moderately decreased T1 signal intensity, mildly high T2 signal intensity, and delayed gadolinium enhancement of the pancreatic parenchyma on post-gadolinium images. Additional findings that may be observed in AIP include low signal capsule-like rim surrounding the diseased parenchyma on T2-weighted images, delayed post-gadolinium enhancement[76], absence of parenchymal atrophy, ductal dilatation proximal to the site of stenosis, absence of peripancreatic fluid, and clear demarcation of the abnormality[77]. The diffuse form of AIP may mimic diffuse disorders like lymphoma, metastases or other diffuse infiltrative processes however, in most of these disorders, the parenchyma is heterogeneous and shows irregular contour.

Focal disease is less common and manifests as a well-defined T2 hyperintense mass with a T2 hypointense capsule-like rim surrounding the diseased parenchyma which may show delayed post-gadolinium enhancement, often involving the head and mimicking pancreatic adenocarcinoma but most often without associated pancreatic ductal cutoff. The characteristic imaging finding in MRCP is focal, segmental or diffuse stenosis or irregularity of main pancreatic duct.

The most commonly involved segment is the intra pancreatic common bile duct, accompanied by a lesser degree of upstream dilatation of the main pancreatic duct. Less frequently, multifocal intrahepatic biliary strictures are also noted in AIP[78].

Groove/paraduodenal pancreatitis: Groove or paraduodenal pancreatitis is an uncommon type of focal chronic pancreatitis; which may affect the groove between the head of the pancreas, the duodenum, and the common bile duct. The rest of the pancreatic parenchyma is slightly compromised or spared[79,80]. Groove pancreatitis is categorized into 2 forms: pure, involving exclusively the groove; or segmental (Figure 21), involving the groove and extending in to the pancreatic head[80]. Pathogenesis remains controversial but may result from obstruction of the accessory pancreatic duct as it drains into the second portion of the duodenum through the minor papilla[81]. It may be due the previous biliary diseases, gastric resections, peptic ulcer disease, true duodenal wall or pancreatic head cysts, or pancreatic head heterotopias in the duodenum[80,82]. Both pure and segmental form can cause progressive stenosis of the pancreatic duct, leading subsequently to diffuse chronic pancreatitis. The histopathologic hallmark of groove pancreatitis is the presence of fibrosis and scar
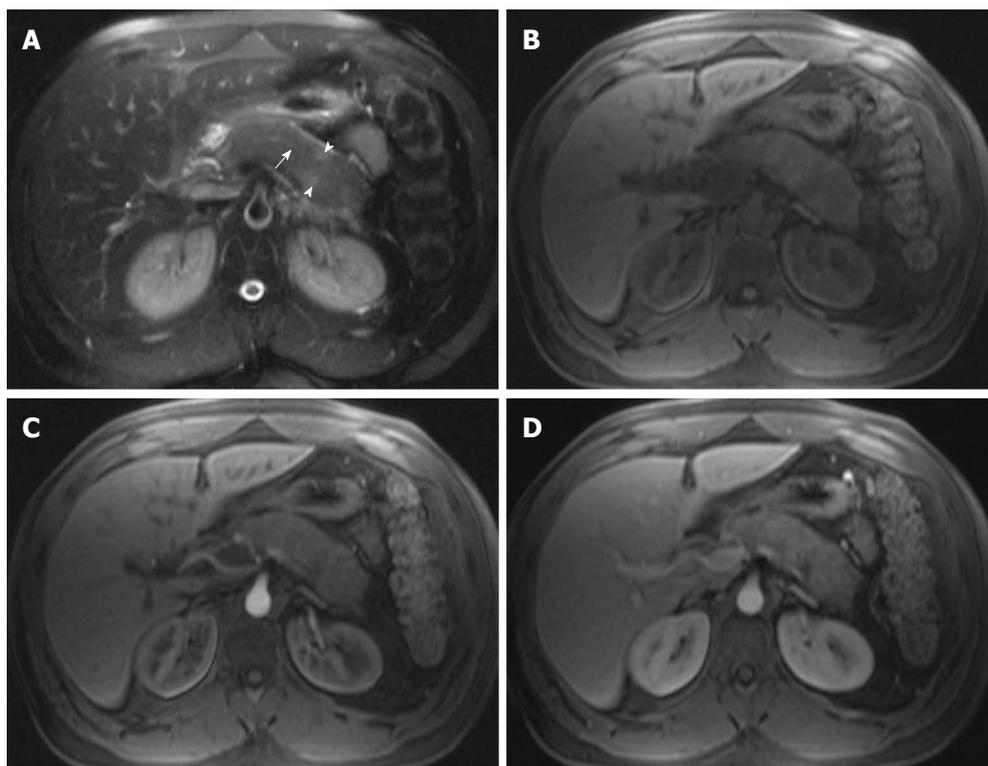
Figure 20 Autoimmune pancreatitis. A: Axial single-shot turbo spin-echo T2-weighted (HASTE) image with fat-suppression; B: Axial T1-weighted image. Post-Gadolinium 3D-GRE T1-weighted images with fat-suppression during the hepatic arterial-dominant (C) and hepatic-venous phases (D). There is evidence of diffuse pancreatic swelling with patchy mildly increased T2 signal (arrowheads) (A), decreased T1 signal (B), loss of the normal pancreatic lobulations, and diffuse obliteration of the pancreatic duct (arrow) (A); associated with patchy decreased early enhancement (C) and progression of enhancement on subsequent phases (D) in keeping with autoimmune pancreatitis.
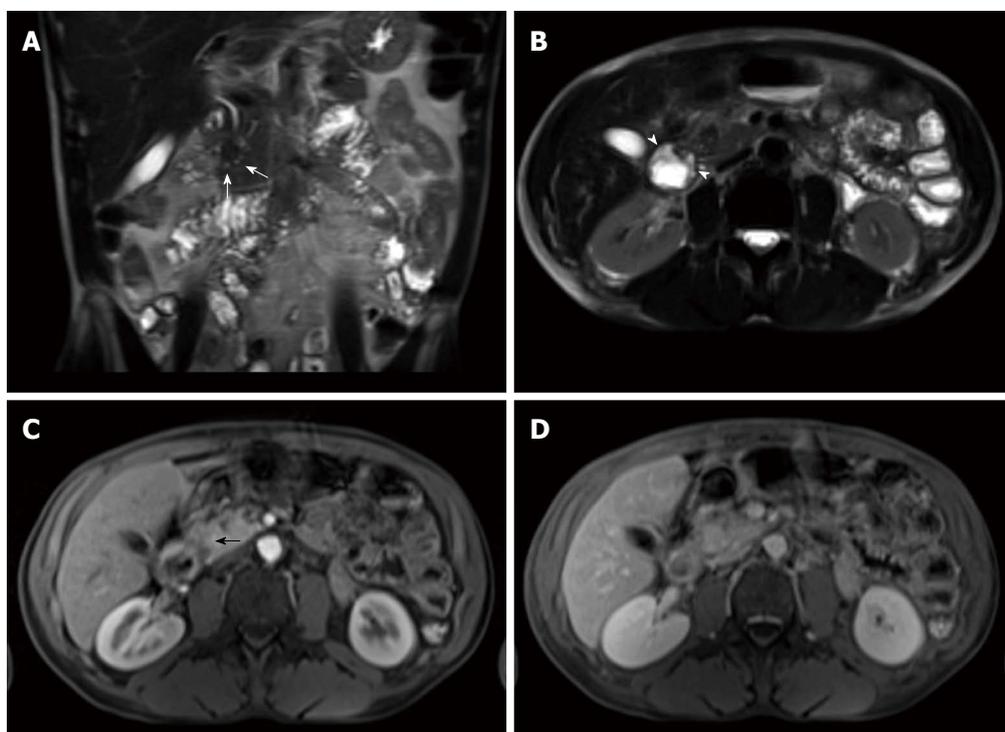
Figure 21 Segmental form groove pancreatitis. Coronal (A) and axial (B) single-shot turbo spin-echo T2-weighted (HASTE) images with fat-suppression. Axial post-Gadolinium 3D-GRE T1-weighted images with fat-suppression during the hepatic arterial-dominant (C) and interstitial phases (D). There is mild duodenal thickening and edema (arrowheads) (A, B) associated with a sheet-like mass in the pancreaticoduodenal groove that extends to the pancreatic head and demonstrates decreased T1 signal pre-contrast (not shown), slightly decreased T2 signal with tiny cystic changes (white arrows) (A, B), early arterial hypo-enhancement (black arrow) (C) and progressive enhancement on the subsequent delayed images (D) due to fibrosis, in keeping with segmental form groove pancreatitis.

tissue within the pancreaticoduodenal groove, in the pure form; or within the groove and superior portion of the pancreatic head, in the pure form of the disease. The most relevant differential diagnosis of groove pancreatitis, particularly in its segmental form, is pancreatic head adenocarcinoma.

The most characteristic finding on MRI is a sheet like mass between the head of pancreas and duodenal C-loop. The mass demonstrates low signal on T1-weighted images compared to the rest of the pancreatic parenchymal tissue, and variable signal on T2-weighted images. This variation in the T2 signal can be attributed to the time of onset of the disease; as subacute form of the disease shows brighter signal on T2-weighted images due to edema, while chronic form of the disease has a lower T2 signal due to fibrosis[82]. Gadolinium-enhanced dynamic images show delayed and progressive heterogeneous enhancement, reflecting the fibrous nature of the tissue. Cystic lesions can also be well seen on T2-weighted images in the groove or duodenal wall[80].

It may be challenging to differentiate groove pancreatitis from pancreatic head duct adenocarcinoma. Recently, it was shown that by using three strict diagnostic criteria for groove pancreatitis: (1) focal thickening of the second part of the duodenum; (2) abnormal increased enhancement of the second part of the duodenum; and (3) cystic changes in the region of the pancreatic accessory duct, distinction from pancreatic duct adenocarcinoma could be achieved with high diagnostic accuracy (87.2% of patients), and a diagnosis of cancer could be excluded with a negative predictive value of 92.9%[83].

Hereditary pancreatitis: Hereditary pancreatitis is an autosomal dominant disease presenting as multiple episodes of pancreatitis in the absence of any predisposing factors and is consider as a premalignant disease[84]. Imaging findings include parenchymal and intraductal calcifications and parenchymal atrophy. However, in hereditary pancreatitis, imaging plays an important role to rule out structural causes of pancreatitis and to closely monitor the development of pancreatic cancer, the risk of which is increased by many folds in these patients.

NEW TECHNIQUES

Continuous development in MRI technologies have resulted in new techniques with increased signal-to-noise ratio, shorter breath-hold scan time and respiratory triggering techniques to produce improved image quality and diagnostic performances. Edelman *et al*[85] reported that there is a marked improvement in SNR at 3 T compared with 1.5 T (by a factor of 2 in some cases) when using identical imaging parameters to image the pancreas. A recent study showed that respiratory-triggered 3D-MRCP using a navigator technique was feasible in routine clinical practice. The navigator technique improved the image quality and lesion visualization of free-breathing 3D-MRCP compared with conventional respiratory triggering techniques using bellows (bellows technique)[86].

Imaging patients unable to hold their breath is challenging. Elderly or very sick patients, who are often part of the pancreatitis population, cannot adequately hold their breath for the required time to acquire T1-weighted images. Radial 3D-GRE delivers robust contrast-enhanced imaging for these patients by being resistant to motion artifacts. A previous study by Bamrungchart *et al*[6] has shown that free-breathing radial 3D-GRE is of value for pancreatic MR imaging in patients who are unable to suspend respiration.

MR spectroscopy with non-invasive *in-vivo* assessment of metabolite concentrations has been applied in a variety of different tissues (brain, prostate, breast, and liver). Hence, spectroscopy of the pancreas has the potential to offer a more accurate tissue characterization[48]. Despite this, Su *et al*[87] characterized the normal pancreas at 3.0 T and identified metabolites such as lipid, choline and cholesterol. Cho *et al*[88] used MR spectroscopy to distinguish between patients with chronic focal pancreatitis and patients with pancreatic carcinoma and found fewer lipids in pancreatitis than in pancreatic carcinoma. Furthermore, other studies also detected differences between normal pancreatic tissue and carcinoma tissue with alterations in lipid, choline and fatty acids[89,90]. However, to the best of our knowledge, this technique has not yet been applied in the characterization of patients with pancreatitis.

CONCLUSION

In this review we emphasized the role of MRI in imaging all types of acute and chronic pancreatitis, pancreatitis complications, and other important differential diagnoses that mimic pancreatitis. MRI is a valuable alternative modality, with at least equal diagnostic performance to CT for the diagnosis and follow-up of acute and chronic pancreatitis. The recent development of new respiratory gating techniques, motion resistant pulse sequences, and additive advantages of MRCP imaging protocols make MRI a very accurate investigation modality for assessing patients with pancreatitis; particularly acutely ill patients unable to breath hold. MRI is a non-ionizing cross sectional imaging method with a safer intravenous contrast profile. This is particularly important in patients with acute pancreatitis, who often have a concomitant renal impairment of some degree and often require repeated follow-up imaging. Additionally, MRI offers higher sensitivity for the diagnosis of subtle early changes of acute pancreatitis (*i.e.*, interstitial pancreatitis and peripancreatic edema) and early manifestations of chronic pancreatitis.

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2014 ADVANCES IN PANCREATITIS

Autoimmune pancreatitis in the context of IgG4-related disease: Review of imaging findings

Leslie K Lee, Dushyant V Sahani

Leslie K Lee, Dushyant V Sahani, Division of Abdominal Imaging and Intervention, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, United States
Author contributions: Lee LK and Sahani DV have each made substantial contributions to the conception, drafting, and revision of the manuscript, and have given final approval of the version to be published.
Author contributions: Leslie K Lee, MD, Division of Abdominal Imaging and Intervention, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, 55 Fruit St., White Bldg. 270, Boston, MA 02114,
United States. llee22@partners.org
Telephone: +1-617-7262000 Fax: +1-617-7262000
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Abstract

Current understanding of autoimmune pancreatitis (AIP) recognizes a histopathological subtype of the disease to fall within the spectrum of IgG4-related disease. Along with clinical, laboratory, and histopathological data, imaging plays an important role in the diagnosis and management of AIP, and more broadly, within the spectrum of IgG4-related disease. In addition to the defined role of imaging in consensus diagnostic protocols, an array of imaging modalities can provide complementary data to address specific clinical concerns. These include contrast-enhanced computed tomography (CT) and magnetic resonance (MR) imaging for pancreatic parenchymal lesion localization and characterization, endoscopic retrograde and magnetic resonance cholangiopancreatography (ERCP and MRCP) to assess for duct involvement, and more recently, positron emission tomography (PET) imaging to assess for extra-pancreatic sites of involvement. While the imaging appearance of AIP varies widely, certain imaging features are more likely to represent AIP than alternate diagnoses, such as pancreatic cancer. While nonspecific, imaging findings which favor a diagnosis of AIP rather than pancreatic cancer include: delayed enhancement of affected pancreas, mild dilatation of the main pancreatic duct over a long segment, the "capsule" and "penetrating duct" signs, and responsiveness to corticosteroid therapy. Systemic, extra-pancreatic sites of involvement are also often seen in AIP and IgG4-related disease, and typically respond to corticosteroid therapy. Imaging by CT, MR, and PET also play a role in the diagnosis and monitoring after treatment of involved sites.

Core tip: The imaging appearance of autoimmune pancreatitis (AIP) varies widely. The literature is reviewed for imaging characteristics that favor a diagnosis of AIP rather than differential considerations such as pancreatic cancer. Response to steroid therapy and the presence of extra-pancreatic lesions are often seen in AIP and in IgG4-related disease. Extra-pancreatic findings and the role of imaging in monitoring their response to therapy are also reviewed, including recent developments in positron emission tomography imaging.

INTRODUCTION

Autoimmune pancreatitis (AIP) was first described by Yoshida *et al*[1] in 1995 as a form of chronic pancreatitis. In the interval, the associated finding of abnormally elevated serum concentrations of IgG4 among AIP patients was first reported in 2001[2], and extra-pancreatic manifestations of disease were first identified among AIP patients in 2003[3]. These and other developments have contributed to the evolution of the understanding of the disease[4] and AIP is now recognized to represent a manifestation of IgG4-related disease[5,6].

AIP IN THE CONTEXT OF IGG4-RELATED DISEASE

IgG4-related disease has been recently-recognized as a systemic inflammatory disorder characterized by stereotypic histopathological features of a dense lymphoplasmacytic infiltrate, "storiform" fibrosis, and obliterative phlebitis[7,8]. It is a systemic process which may involve one or multiple organs, either synchronously or metachronously. IgG4-related disease has been described in virtually every organ system, including the pancreas[5,6], demonstrating common histopathological findings. As a result, a host of organ-specific pathologies previously thought to be unrelated are now recognized in the spectrum of IgG4-related disease, including: salivary glands (Mikulicz's syndrome), thyroid gland (Riedel's thyroiditis), orbit (orbital pseudotumor), aorta (non-infectious/inflammatory aortitis or periaortitis), pancreas (AIP), retroperitoneum (Ormond's disease or retroperitoneal fibrosis), and kidneys (tubulointerstitial nephritis).

Two types of AIP, 1 and 2, are presently recognized, found to share overlapping histopathological and clinical characteristics, but also important differences[9-11]. Of note, while Type 1 disease demonstrates IgG4-related infiltrates and serologic abnormalities, these features are absent in Type 2 disease. Additionally, extra-pancreatic organ involvement and disease relapse are associated with Type 1 and not Type 2 disease[12]. International consensus diagnostic criteria have been established for AIP, predicated on clinical, laboratory, imaging, and histopathologic data. In addition to characteristic histopathological findings, diagnostic characteristics of AIP include abnormal elevations of serum IgG4 levels, extra-pancreatic organ involvement, and responsiveness to a trial of corticosteroids. By imaging, while certain features are considered diagnostic, Types 1 and 2 cannot be reliably distinguished[9,11].

Demographics

An uncommon entity, the global burden of IgG4-related disease is difficult to assess, a problem made more challenging by its evolving characterization encompassing various organ-based pathologies which were previously thought to be disparate. However, population-based epidemiological data are available relating to AIP in Japan,

where estimates based on national survey data estimate the prevalence of AIP as 0.82-2.2 per 100000 individuals[13,14]. The disease typically involves men more than women, at a ratio of 2.9-3.7 to 1, and typically involves individuals older than 50 years of age. Pertaining to AIP, groups around the world have also reported on their clinical experience[15-18].

Diagnostic features of IgG4-related disease

The diagnosis of IgG4 disease relies on the synthesis of clinical, laboratory, radiologic and histopathologic findings[5,9,11,12]. National consensus criteria for diagnosis from Japan[19] are comprised of two central, specific, findings: the first, of abnormally elevated serum IgG4 concentration > 135 mg/dL; and the second, in histopathologic analysis, of > 40% of IgG+ plasma cell positive for IgG4, and > 10 IgG4+ cells per high power field. Additional clinical, laboratory, and histopathological findings may be less specific, but increase the sensitivity for detection of organ-specific pathology in the IgG4-related disease spectrum.

Clinically, IgG4-related disease typically presents in subacute fashion. Most patients are not constitutionally ill, and fever as a symptom is unusual; the myriad clinical presentations of IgG4-related disease have previously been summarized[5]. Symptoms are typically nonspecific, and further investigations are typically necessary before the diagnosis is reached. Laboratory evaluation for IgG4-related disease has centered on serum concentration of IgG4, since this finding was first reported in AIP patients in 2001[2]. However, elevated serum IgG4 levels are detected in other types of immune-mediated and allergic disorders, as well as in infectious and malignant conditions[20]. Nonetheless, the generally accepted upper limit of normal of serum IgG4 concentration is 135 mg/dL; levels elevated beyond this are considered abnormal, including in the Japanese national consensus criteria. It should be noted that serum IgG4 abnormalities are not seen in Type 2 AIP, and at the diagnostic threshold of 135 mg/dL, up to 30% of patients with IgG4-related disease may have normal serum IgG4 levels[21].

Given the nonspecific nature of presenting symptoms, laboratory and radiologic investigation present complementary data in reaching a diagnosis of IgG4-related disease. Imaging may be of particular utility in identifying focal abnormalities that may represent biopsy targets. Even so, the characteristic of the disease to form tumefactive lesions often necessitates biopsy to exclude a malignant or neoplastic process.

IMAGING FINDINGS OF AIP

Cross-sectional imaging findings of AIP were initially described in 1998[22,23]. Clinical investigators since then have reported on the imaging appearance of AIP by a multitude of imaging characteristics, including morphology of the pancreatic parenchyma and main pancreatic duct, associated tissue (fat, lymph nodes), signal, and response

Figure 1 Contrast-enhanced computed tomography findings in autoimmune pancreatitis. A: Enlargement of the distal pancreatic body and tail (between arrowheads), with fine peri-pancreatic stranding of the adjacent fat (small arrow); B: The "capsule" or "rim" sign, a hypo-attenuating rim encircling the anterior and posterior margin of the pancreas (white arrows); C: Multifocal main pancreatic duct narrowing (black arrow).

to administration of intravenous contrast agents. Modalities employed by investigators include cross-sectional techniques of computed tomography (CT) and magnetic resonance (MR) imaging, endoscopic techniques such as endoscopic retrograde cholangiopancreatography (ERCP) and endoscopic ultrasound (EUS), and more recently, positron emission tomography (PET).

CT and MR imaging

Morphologic and signal characteristics: The CT appearance of AIP was first described in 1998. In two case series' of five and three patients[22,23], CT demonstrated a diffusely enlarged pancreas in all patients. Since then, studies of patients with AIP have demonstrated heterogeneity of the morphologic presentation on both CT and MR imaging: diffuse enlargement has been shown among 11%-56% of patients; focal or mass-like enlargement among 28%-59% of patients (Figure 1A); and no enlargement, or a normal appearance of the pancreas, in a minority of patients, 9%-16%[15,16,24,25]. In another series, investigators also characterized a 'mixed' appearance of diffuse and focal enlargement in 56% of 36 patients[25].

Peri-pancreatic fat planes are typically preserved on cross-sectional imaging[23]. Minimal peri-pancreatic stranding (Figure 1A), without vascular encasement, parenchymal calcification, or peripancreatic fluid collection, was seen in six patients with diffuse pancreatic enlargement, in a study of 25 patients with AIP[15]. By comparison, in that study, an accessory finding more commonly observed was one of enlarged peripancreatic lymph nodes. Imaging presentation with acute pancreatitis has rarely been reported: in one series of imaging findings on 22 patients with AIP, the authors noted the appearance of one case consistent with acute pancreatitis[16].

By MR imaging, signal abnormality representing AIP typically demonstrates relative T1 hypo-intensity, and relative T2 hyper-intensity[22,25,26]. Recent studies have sought to distinguish AIP from differential considerations such as pancreatic cancer using MR diffusion characteristics, and other imaging features (Section 4, below).

Enhancement characteristics: Classically, upon administration of intravenous contrast material, AIP demonstrates diminished enhancement in the early, or arterial phase, and relatively increased or prolonged enhancement in the delayed or venous phase (Figure 2)[22,23]. Despite variation in acquisition and definition, subsequent studies have typically supported this pattern of enhancement by both CT[27,28] and MR[24] imaging. Takahashi *et al*[27] quantitatively assessed dual-phase contrast enhanced CT among 43 AIP patients and 25 patients with normal pancreas. In the pancreatic phase, the mean CT attenuation of pancreatic parenchyma among AIP patients (85 HU) was significantly lower than that among the control group (104 HU). Delayed enhancement, defined as a 15HU or greater increase from the pancreatic phase to the hepatic phase, was observed in seven of the 13 patients (54%) with focal AIP. In separate study of imaging related to 36 patients with AIP comprising 86 contrast-enhanced CT and MRI scans[25], investigators noted hypo-enhancement in the arterial phase in 58% and 52% for CT and MRI, respectively. In that study however, delayed enhancement was found to be significantly more pronounced by MR imaging: whereas 75% of late-venous phase enhancement in CT was iso-attenuating, 74% of late-venous enhancement was hyper-enhancing by MR.

An early report[22] also noted that on CT, in 4 of 5 patients, "a capsule-like low density rim surrounded the pancreas on both early and delayed [contrast-enhanced] images," giving rise to the "capsule" or "rim" sign of AIP (Figure 1B). The correlate on MR imaging is of a T1 and T2 hypo-intense rim, with delayed enhancement, demonstrated in three of four patients. The sensitivity of this finding for AIP has been subsequently shown to be generally low for both CT and MR imaging, ranging from 12%-40%, but may potentially distinguish AIP from pancreatic malignancy[15,24-27,29].

Endoscopic techniques: ERCP and EUS

Abnormality of the intra- and extra-hepatic biliary system, including the main pancreatic duct (MPD), is common in AIP. MPD involvement varies widely, and may demonstrate irregular narrowing, in either a diffuse or segmental distribution (Figure 3)[26]. In one series of 20
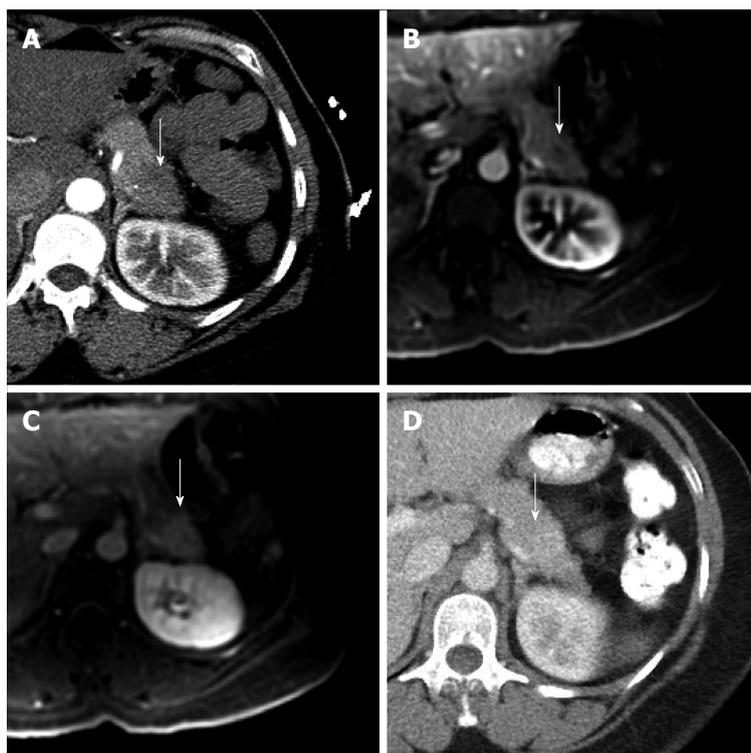
Figure 2 Delayed enhancement on computed tomography and magnetic resonance imaging in autoimmune pancreatitis. A, B, C: Focal autoimmune pancreatitis in the pancreatic tail (white arrow) with delayed early arterial enhancement on arterial phase computed tomography (CT) (A) and magnetic resonance (B, fat-saturated T1-weighted image, 30 s post-injection), with subsequent delayed enhancement (C, fat-saturated T1-weighted image, 180 s post-injection); D: Follow-up CT after corticosteroid therapy demonstrating resolution of prior enhancement abnormality (white arrow).
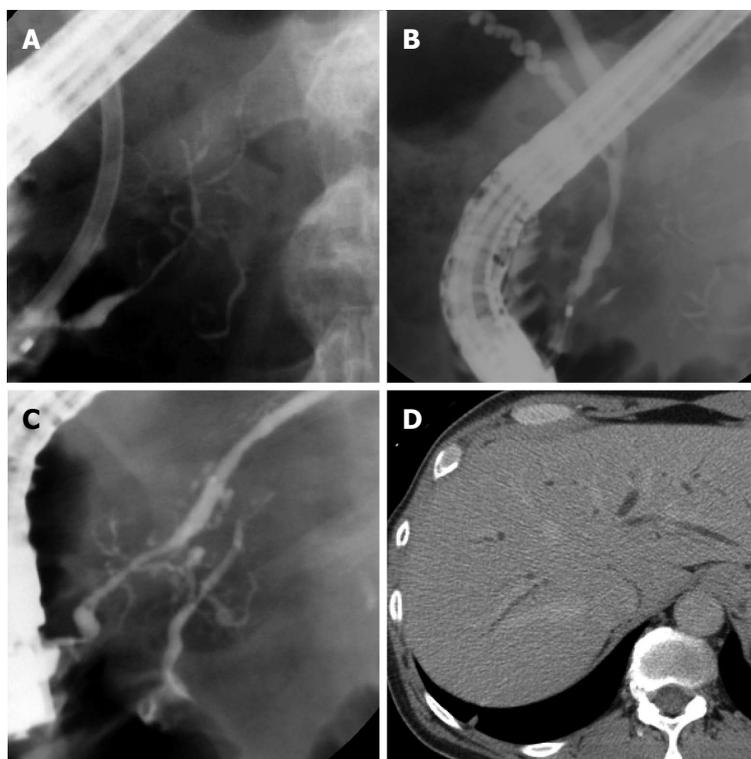
Figure 3 Biliary involvement in autoimmune pancreatitis. A, B, C: Endoscopic retrograde cholangiopancreatographydemonstrating diffuse narrowing of the main pancreatic duct (A) and segmental narrowing of the lower common bile duct (B), with improvement of main pancreatic duct narrowing after therapy (C); D: Computed tomographic image demonstrating intrahepatic biliary ductal dilatation in a patient with biliary involvement from IgG4-related disease.

patients who underwent ERCP, diffuse narrowing was found in six patients (30%), and segmental narrowing was found in nine patients (45%)[26]. The length of MPD narrowing was longer than 3 cm in 18 patients (90%). In another series of 19 patients who underwent ERCP, diffuse irregularity and narrowing of the MPD was observed in nine patients (47%), while focal stricture in the proximal MPD was seen in six patients (32%)[15]. Biliary duct abnormalities were seen in 16 of 19 patients who underwent ERCP (84%). The most common abnormality was stricture of the distal common bile duct, present in 12 patients (60%), while multiple short-segment intrahepatic duct strictures were present in six patients (30%). Biliary involvement varies widely, and multifocal narrowing of the MPD (Figure 1C) and narrowing of the lower common bile duct have been reported in as high a proportion as 85% and 90%, respectively[30]. Readers are additionally referred to a recent review for detailed discussion of IgG4-related sclerosing cholangitis, which has overlapping features[31].

The sensitivity of ERCP to diagnose AIP is limited, but can be improved with directed training of key features. A multicenter, international study[32] identified four key features of AIP from a series of 20 patients: long stricture (greater than one-third the length of the MPD); upstream dilatation from the stricture less than 5 mm; multiple strictures; and side branches arising from a strictured segment. Following training with a teaching module principled upon these features, the sensitivity of an international group of physicians to detect AIP increased significantly from 44% to 71%, with specificity of 83%.

Consistent with the varied morphologic presentation of AIP seen on CT and MR imaging, endoscopic ultrasound may reveal diffuse enlargement of the pancreas with altered echotexture, or may demonstrate a focal hypoechoic mass[33]. In one study among 21 patients who underwent EUS[15], diffuse enlargement with altered echotexture was seen in 13 patients (62%), while six patients had focal enlargement of the head of the pancreas (29%).

Magnetic resonance cholangiopancreatography

Magnetic resonance cholangiopancreatography (MRCP) can provide complementary data in the diagnosis of AIP and assessment of MPD involvement, depending on the pattern of involvement[26]. In a series of 20 AIP patients, MRCP findings were compared with ERCP findings[34]. Among patients with focal AIP, the narrowed portion of the MPD was not visualized, while among patients with diffuse AIP, the MPD was incompletely visualized or not visualized. In the latter setting, non-visualization of the MPD may limit detection of duct involvement, yet MRCP may still be helpful in follow-up after therapy. In a separate study comparing MRCP findings among cohorts of 38 AIP patients, 40 pancreatic cancer patients, and 40 normal controls, ERCP was used as the gold standard[35]. The authors found MRCP to be 65% accurate (22 of 34 patients) for depicting MPD morphology among patients

with AIP, significantly less than that of the cohort of patients with pancreatic cancer (89%, 23 of 26 patients) or those with normal pancreas (100%, 40 of 40 patients).

Angiography and peripancreatic vascular findings

Angiographic findings related to AIP were reported by Kamisawa *et al*[36] in 2003. Among 13 patients with AIP, angiography demonstrated irregular narrowing of the anterior superior pancreaticoduodenal artery in seven (54%) and of the posterior superior pancreaticoduodenal artery in 4 patients (31%). Deviation of the portal or splenic vein was observed in 4 cases (31%); collateral venous circulation was observed on account of stenosis or obstruction in 3 cases (23%). The presence of irregular narrowing of the pancreatic arteries similar to encasement sometimes detected in pancreatic carcinoma; these angiographic findings can cause confusion in the diagnosis of AIP.

Subsequent studies with cross sectional imaging have reported similar rates of peripancreatic vascular involvement. Takahashi *et al*[37] reported vascular involvement in 11 of 25 (44%) of AIP patients. Raina *et al*[38] demonstrated splenic vein and/or artery involvement was seen in six of 26 patients (23%). Vlachou *et al*[39] noted narrowing of the splenic vein with collateral vessel formation was seen in 9 of 57 patients (16%), with normalization of vessel caliber following resolution of AIP. Ishikawa *et al*[40] reviewed CT imaging among 54 AIP patients, finding 24 cases (44%) which demonstrated peripancreatic vascular involvement, with stenosis or occlusion of the splenic vein in 22 cases, of the superior mesenteric or portal vein in 13 cases, and development of collaterals in 18 cases. Among 16 patients who underwent steroid therapy, 14 demonstrated improvement in vascular involvement (87%).

Positron emission tomography

PET, typically used in clinical oncology to localize areas of normal or abnormal physiology based on uptake of radiopharmaceutical imaging agents[41] has found useful application in the imaging of inflammatory disease[42]. PET imaging following intravenous administration of a radiopharmaceutical such as 2-(18)F-fluoro-2-deoxy-d-glucose (^{18}F-FDG), either alone or in combination with concurrent CT imaging (PET/CT), allows for whole body imaging to identify areas of abnormally increased cellular metabolism[43].

With regard to AIP, ^{18}F-FDG uptake at pancreatic and extra-pancreatic lesions have been shown in case reports of AIP/IgG4-related disease since 1999[44-47]. Nakamoto *et al*[44] initially described two cases of AIP demonstrating diffusely and focally intense pancreatic uptake, with resolution after steroid therapy. Kajiwara *et al*[47] described two cases with multifocal ^{18}F-FDG uptake of the pancreas, corresponding to focal pancreatic masses of AIP. Kawamura *et al*[45] and Sato *et al*[46] additionally reported extrapancreatic findings in cases of AIP associated with sclerosing cholangitis, sialadenitis, and lymphadenopathy.

Table 1 Imaging findings favoring a diagnosis of autoimmune pancreatitis rather than pancreatic cancer

Diffuse pancreatic enlargement
Delayed enhancement of affected pancreas
Long segment MPD narrowing
MPD dilatation not in excess of 4-5 mm
Multiple sites of MPD narrowing
"Capsule" sign
"Penetrating duct" sign
Low ADC value reflecting restricted diffusion on diffusion weighted MR imaging[51,52]
Improvement of findings following short course of corticosteroid therapy

MPD: Main pancreatic duct; ADC: Apparent diffusion coefficient.

As with its accepted application in clinical oncology, in the context of IgG4-related disease, ^{18}F-FDG PET may prove valuable in providing complementary data in the delineating the extent of organ involvement, staging the extent of disease, guiding biopsy early in the diagnostic evaluation, and monitoring response to therapy[42].

DISTINCTION OF AIP FROM PANCREATIC MALIGNANCY

The varied appearance on cross-sectional imaging of AIP can make for a diagnostic quandary. For example, in a case series of the early clinical experience encompassing 37 patients with AIP between the years 1989 and 2005[48], 6 patients had been initially misdiagnosed with pancreatic cancer, and two patients had been initially misdiagnosed with biliary malignancy. Authors noted that 5 cases were misdiagnosed on account of the non-existence of, or unfamiliarity with, the entity of AIP. In another early report, 9 patients among a series of 17 patients with AIP were initially suspected to have pancreatic cancer[36]. The authors cited a number of variables of the cohort that raised concern for pancreatic malignancy, including: demographics (14 patients were male, 16 patients older than 60 years), clinical presentation (jaundice in 13 patients), serum studies (9 patients had elevated tumor markers), and radiologic evidence of biliary duct stenosis (16 patients).

Given the potential of overlapping clinical and radiologic presentations of AIP and important differential considerations such as pancreatic malignancy, numerous subsequent investigations have sought to discern AIP from a malignant etiology. In an early study, investigators retrospectively compared findings from nine patients with focal AIP with 80 patients with pancreatic cancer, and 11 patients with alcohol-related pancreatitis[49]. Significant factors differentiating focal AIP from pancreatic cancer included: homogeneous delayed enhancement on contrast-enhanced CT, and ERCP findings of long-segment stenosis of the MPD, and a lesser degree of MPD dilatation proximal to stricture. Other groups have subsequently employed clinical and radiologic means to differentiate AIP from pancreatic cancer, using CT, MR

and PET imaging, and the imaging response to a trial of steroid therapy in diagnostic protocols[11,50]. Imaging features favoring a diagnosis of AIP rather than pancreatic cancer are summarized in Table 1.

Signal and enhancement characteristics

Discerning imaging features of AIP *vs* pancreatic cancer include morphology, attenuation, signal, and enhancement characteristics, and certain specific signs ("capsule" and "penetrating duct" signs).

International consensus guidelines recognize diffuse pancreatic enlargement with delayed enhancement to represent typical findings of AIP[11]. Quantitatively, CT studies on enhancement patterns of pancreatic AIP lesions *vs* malignancy have demonstrated mean CT attenuation in the delayed or hepatic phase of imaging to be significantly greater in AIP than in pancreatic cancer[27,51]. Contrasting data were reported however regarding enhancement in the early or pancreatic phase, possibly due to differences in contrast administration and timing. Among those two studies, Takahashi *et al*[37] found peri-pancreatic stranding and calcifications significantly associated with AIP, while Muhi *et al*[51] observed that the frequency at which calcifications were seen was not statistically significant.

The capsule sign of AIP, as previously described (Figure 1B), while of variable sensitivity, favors a diagnosis of AIP rather than pancreatic cancer when present: studies have shown the capsule sign is significantly more frequently associated with AIP[37,51,52], and rarely reported in pancreatic cancer.

The finding of greater delayed enhancement in AIP (Figure 2B and C) was demonstrated on MR imaging by Hur *et al*[52]. Two groups were assessed at the lesion level, 14 among AIP patients, 28 among pancreatic cancer patients. There was significantly greater delayed enhancement at 3-min post contrast administration in the AIP group (10/14, 71%) in the AIP group compared to the pancreatic cancer group (57%). Signal intensity in the arterial and portal venous phase following contrast administration did not differ significantly.

Using MR imaging, other investigators have sought to discern AIP from pancreatic cancer *via* diffusion weighted sequences. In diffusion weighted MR imaging, the apparent diffusion coefficient (ADC) can be calculated as a measure of free diffusion of assessed water molecules; lower ADC values indicate restricted diffusion[53]. Histopathological correlation of tissue with ADC values bear an inverse association of ADC value and cell density, *i.e.* low ADC values are associated with tissue of high cell density[54]. Early AIP data using diffusion weighted imaging demonstrated significantly decreased ADC in AIP cases, compared to cases of chronic alcoholic pancreatitis and normal controls[55]. Subsequently, investigators have quantitatively shown that ADC values are significantly lower in AIP than in pancreatic cancer. Following steroid therapy among AIP patients, foci of restricted diffusion decreased markedly or resolved, with ADC values increasing almost to that of normal pancreas[51,52,55,56].

In receiver-operating curve analysis, Hur *et al*[52] found that a threshold ADC value of 1.26×10^{-3} mm^2/s, below which would distinguish AIP from pancreatic cancer, yielded a sensitivity of 83.3% and a specificity of 79.2%. Similarly, in sensitivity analysis of ten patients with AIP and 70 patients with pathologically proven pancreatic carcinoma, Muhi *et al*[51] applied two criteria in tandem, delayed enhancement and ADC less than 0.88×10^{-3} mm^2/s, to suspected cases of focal AIP, achieving sensitivity and specificity of 100%.

Main pancreatic duct involvement

Dilatation of the MPD may be seen in both AIP and pancreatic cancer. However, AIP demonstrates a lesser degree of MPD dilatation by both conventional and MR/MRCP imaging than that seen in pancreatic cancer, typically less than 4 mm[34,35,51,52]. This pattern reflects that seen by ERCP, where AIP typically demonstrates long segment narrowing over a segment greater than 3 cm (Figure 3A), with upstream dilatation less than 4 mm[49]. In receiver-operating curve analysis conducted by Muhi *et al*[51], the group found that a threshold value of 4 mm of upstream MPD dilatation on MRCP yielded sensitivity of 100% and specificity of 76% for AIP. Additionally, multiple sites of MPD narrowing (Figure 1C) favor the diagnosis of AIP rather than pancreatic cancer, as per international consensus guidelines[11,35]. Complete obstruction of the MPD and abrupt cut-off of the MPD however, are findings differentially associated with pancreatic cancer rather than AIP[37,51].

Studies have also evaluated the value of the 'penetrating duct sign' in differentiating AIP from pancreatic cancer. Initially associated with ultrasound or ERCP findings of focal pancreatitis[57], this sign represents the finding of a non-obstructed MPD penetrating a focal pancreatic mass lesion. Ichikawa *et al*[58] previously assessed the penetrating duct sign on MRCP to have high specificity in determining inflammatory pancreatic mass lesions, and for distinguishing AIP from pancreatic cancer. Carbognin *et al*[24] found the penetrating duct sign to be present in 6 of 14 AIP cases (43%) by secretin-MRCP. In studies comparing cohorts of AIP patients and pancreatic cancer patients, MRCP studies have found the penetrating duct sign to be of variable sensitivity, but with high specificity for AIP when present. Hur *et al*[52] observed the penetrating duct sign in 3 of 9 AIP patients (33%) and in none of 29 pancreatic cancer patients (0%). Muhi *et al*[51] observed the penetrating duct sign in 8 of 11 AIP patients (73%) and in 3 of 70 pancreatic cancer patients (4%).

Advanced endoscopic techniques, such as intraductal ultrasound may further discern the etiology of existing stricture, whether from mass effect, edema, or wall thickening; Hirano *et al*[59] demonstrated advanced intrapancreatic biliary wall thickening was associated with increased severity of stricturing. Finally, EUS-guided fine needle aspiration with a 19-gauge needle allows for minimally-invasive tissue sampling, and is commonly used to exclude pancreatic malignancy[60]. Endoscopic techniques and devices specific to IgG4-related disease have been recently reviewed[61].

^{18}F-FDG PET findings

As ^{18}F-FDG PET imaging also has high sensitivity for pancreatic cancer[62], investigators have also evaluated the ability of ^{18}F-FDG PET imaging to differentiate AIP from pancreatic cancer[63-66]. Ozaki *et al*[63] detected ^{18}F-FDG uptake in all 15 patients (100%) with autoimmune pancreatitis, compared to 19 of 26 patients (73%) with pancreatic cancer. Lee *et al*[64] detected ^{18}F-FDG uptake in 17 of 17 AIP patients (100%), *vs* 124 of 151 (82%) of patients with pancreatic cancer. Shigekawa *et al*[65] compared ^{18}F-FDG PET between 18 patients with AIP and 20 patients with pancreatic cancer, with uptake observed in 16 (89%) and 18 (90%) patients, respectively. Described patterns of uptake favoring AIP rather than pancreatic cancer include: diffuse pancreatic uptake, multiple foci of pancreatic uptake, elongated shape of focal uptake (*vs* a nodular pattern of uptake), and heterogeneous uptake (*vs* a homogeneous pattern of uptake)[63,64]. Extra-pancreatic ^{18}F-FDG uptake at the lacrimal glands, salivary glands, thoracic lymph nodes, biliary duct, kidneys, retroperitoneal space, and prostate have been observed in cases of AIP[63-66].

Overall, studies have demonstrated high sensitivity of ^{18}F-FDG PET among patients with AIP, as well as in patients with pancreatic cancer. Extra-pancreatic foci of ^{18}F-FDG uptake may represent associated lesions in IgG4-related disease, or metastatic foci in pancreatic cancer; the role of ^{18}F-FDG PET imaging in the staging of IgG4-related disease is discussed below. While the existing literature suggest certain patterns of uptake that favor one diagnosis *vs* another, correlative clinical and histopathological data remain essential to the course of management.

IMAGING RESPONSE TO CORTICOSTEROID THERAPY

AIP has been widely shown to be responsive to corticosteroid therapy[4,5,10-12]. Imaging plays a role both in diagnostic protocols that aim to discern AIP from pancreatic cancer by the response to a course of corticosteroids, as well as in the assessment of response to therapy.

Improvement, if not complete resolution, of imaging abnormalities in AIP is commonly seen after steroid therapy. Manfredi *et al*[67] specifically evaluated CT examinations of 21 patients with AIP were reviewed before and after steroid therapy. Notably, baseline studies demonstrated hypo-attenuation of affected parenchyma in 19 patients (90%), contrast enhancement abnormality with contrast material retention at the portal venous phase in 18 (86%) patients and contrast material washout in three (14%), and non-visualized of the MPD within affected parenchyma in all patients (100%). Following steroid therapy, CT demonstrated size reduction of affected pancreatic parenchyma, normalization of pancreatic enhancement in 15 (71%), and normalization of the ap-
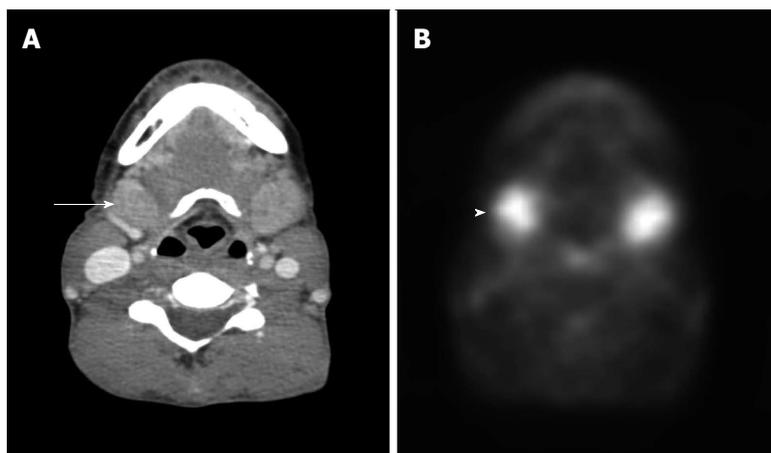
Figure 4 Head and neck findings in IgG4-related disease demonstrated by 2-(18)F-fluoro-2-deoxy-d-glucose positron emission tomography/computed tomography. A: Bilateral, enlarged submandibular glands on computed tomography (arrow); B: Corresponding intense 2-(18)F-fluoro-2-deoxy-d-glucose uptake at the submandibular glands (arrowhead).

pearance of the MPD at affected areas.

Sahani *et al*[68] assessed follow-up CT imaging of 15 AIP patients for imaging factors associated with complete *vs* partial clinical response after steroid therapy. Complete response to treatment was associated with baseline features of diffuse pancreatic parenchymal involvement, and peri-pancreatic stranding. By comparison, partial response was associated with cases with persistent ductal stricture and persistent focal mass-like swelling after resolution of diffuse changes.

Typically, normalization of ^{18}F-FDG uptake abnormalities has also been observed by PET imaging following steroid therapy. In the series reported by Lee *et al*[64], follow-up PET/CT after steroid therapy was performed for eight patients with AIP, whereby residual intense FDG uptake was not observed in each of the eight patients. Matsubayashi *et al*[69] reported on findings of 11 AIP cases with PET imaging both before and three months after the initiation of steroid therapy. ^{18}F-FDG uptake was analyzed semi-quantitatively *via* measure of standardized uptake value (SUV). The mean of maximum SUV among pancreatic lesions differed significantly with therapy, decreasing from 5.12 at baseline to 2.69 following therapy ($P < 0.001$). By the group's SUV criteria, FDG uptake resolved completely in 6 patients (55%), diminished to a faint level in 2 patients (18%), diminished but remained abnormal in 2 patients (18%), and increased after steroid therapy in 1 patient (9%).

Repeat imaging following a trial of steroid therapy of two weeks' duration is recommended in the setting of a new AIP diagnosis, according to international consensus guidelines[11]. Moon *et al*[70] reported imaging (contrast-enhanced CT and ERCP/MRCP) results following a two-week course of steroid therapy among 22 patients with indeterminate imaging for AIP *vs* pancreatic cancer. After the two-week trial, surgical intervention was performed where reduction of pancreatic mass or MPD narrowing was not observed; each of the seven patients who did not demonstrate an imaging response were subsequently diagnosed with pancreatic cancer. Similarly, in the series of Shigekawa *et al*[65], follow-up PET was performed in six AIP patients and in three pancreatic cancer patients, and maximum SUV at follow-up was recorded within one week in five AIP patients and in all three pancreatic cancer patients. In four AIP patients, the change in maximum SUV was greater than 10%, while this value was increased or within 10% of baseline in the three patients with pancreatic cancer.

IMAGING OF IGG4-RELATED DISEASE: EXTRA-PANCREATIC FINDINGS

The observation of extra-pancreatic abnormalities among patients with AIP contributed to the understanding of IgG4-related disease[3,5]. The imaging of extra-pancreatic findings of IgG4-related disease has been reviewed previously[28,39]. Extra-pancreatic organs that may be involved include: the biliary tree, gallbladder, kidneys, retroperitoneum, mesentery, thyroid, lacrimal glands and orbits, salivary glands, lymph nodes, lungs, gastrointestinal tract, and large and medium-caliber arteries (Figures 4 and 5). In a large retrospective series of cross-sectional imaging of 90 patients with AIP, extra-pancreatic lesions were detected in 92% of cases[71]. Extra-pancreatic imaging abnormalities included: hilar lymphadenopathy (78%), wall thickening of bile ducts (78%), peri-pancreatic or para-aortic lymphadenopathy (56%), lung lesions (51%), swelling of lachrymal and salivary gland lesions (47%), retroperitoneal fibrosis (20%), renal lesions (14%), and mass lesions of the ligamentum teres (2%).

While the majority of reports on extra-pancreatic findings of IgG4-related disease center on conventional cross-sectional modalities such as CT and MR, radiopharmaceutical imaging, predominantly with ^{18}F-FDG PET but also with gallium-67, has also been reported. In the case of gallium-67, a case series among 24 AIP patients demonstrated high pancreatic uptake in
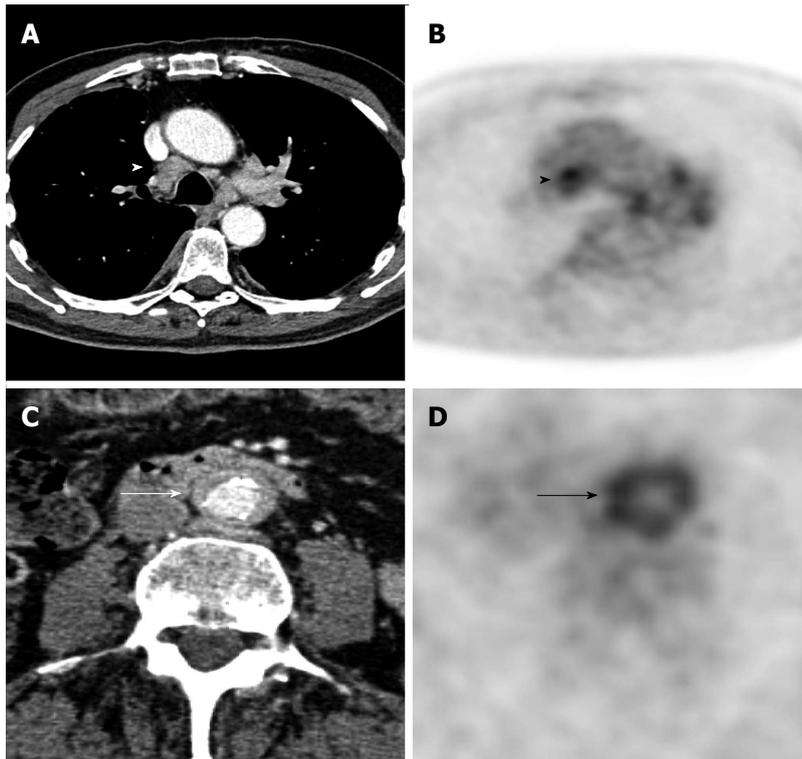
**Figure 5 Thoracic and abdominal findings in IgG4-related disease demonstrated by 2-(18)F-fluoro-2-deoxy-d-glucose positron emission tomography/computed tomography.** A, B: Paratracheal mediastinal lymphadenopathy on computed tomography (CT) (A, white arrowhead) and positron emission tomography (PET) (B, black arrowhead); C, D: Retroperitoneal fibrosis on CT (C, white arrow) and PET (D, black arrow).

16 patients (67%), which resolved after corticosteroid therapy[72]. Pancreatic uptake was significantly associated with elevated serum IgG4 levels, as was hilar gallium-67 uptake. In a series of 13 patients who underwent gallium-67 imaging, high uptake was detected in the pancreas, bilateral hila, salivary glands, lacrimal glands, and periaortic lesions in 10 (77%), 10 (77%), 7 (54%), 7 (54%), and 2 (15%) patients, respectively[73]. Compared with gallium-67, imaging with ^{18}F-FDG is more commonly performed and reported on account of its favorable dosimetry and signal localization characteristics, and is discussed in further detail below.

Renal findings
Certain extra-pancreatic findings have been specifically investigated among patients with AIP. A retrospective study of 2007 investigated renal findings on CT and MRI among patients with AIP[74]. Of 40 patients with CT or MR imaging at presentation, 14 (35%) had renal involvement (12 with parenchymal involvement and 5 with extra-parenchymal involvement). Renal parenchymal lesions had decreased enhancement, and appeared as small peripheral cortical nodules, as round or wedge-shaped lesions, or as diffuse patchy involvement. Thirteen patients with underwent a follow-up study; renal lesions in 10 patients (77%) regressed (9 after steroid treatment, 1 spontaneously) but progressed in three patients without steroid treatment.

In another study of 18 patients with AIP and no his-tory of renal disease, seven patients were found to have renal involvement (39%)[75]. In 4 patients, lesions appeared as multiple renal parenchymal nodules showing decreased enhancement; in 2 cases, diffuse thickening of the renal pelvis wall was seen; in 1 patient, an ill-defined low-attenuation mass-like lesion was identified. None of the lesions was visible on non-contrast-enhanced CT scan. In each of these seven patients, renal lesions regressed after steroid treatment (100%).

Head and neck findings
Pertaining to the head and neck, IgG4-related disease may affect a variety of sites[76], but typically are iso- to hypo-intense on T2-weighted MR imaging. Affected sites include: salivary glands, lacrimal glands, orbits, thyroid gland, lymph nodes, sinonasal cavities, pituitary gland, and larynx (Figure 2). Multiples sites are typically involved. CT imaging of involved organs may demonstrate enlargement or decreased attenuation. MR findings vary, but lesions typically have relatively low signal T2-weighted signal intensity on account of increased cellularity and fibrosis. A retrospective study of 17 patients with IgG4-related disease of the head, neck and brain demonstrated the following distribution of abnormalities: parotid gland 14 (82%), submandibular gland 10 (59%), lacrimal gland 7 (41%), pterygopalatine fossa 3 (18%), pituitary gland 2 (12%), and skull base dura mater 2 (12%)[77]. Lesions presented as either an enlarged gland or glands, or as focal nodules or masses. All lesions were well-defined, showed

homogeneous enhancement, and appeared iso- to hypo-intense on T2-weighted MR imaging. No lesion showed vascular occlusion or compression, or destruction of adjacent bony structures. In a separate study of 15 patients with IgG4-related disease of the head, neck and brain[78], the distribution was as follows: lacrimal gland 8 (53%), cranial nerve involvement 7 (47%), with the infraorbital nerve involved in 4, orbital pseudotumor 5 (33%), and pituitary gland 5 (33%). All lesions were hypo-intense on T2-weighted MR images.

^{18}F-FDG PET imaging in IgG4-related disease

Extra-pancreatic findings have been described by ^{18}F-FDG PET imaging in IgG4-related disease in case reports[79-82] and case series' (Figures 2 and 3)[69,83,84]. In one study of six patients with AIP, whole-body ^{18}F-FDG PET or PET/CT examinations were reviewed at baseline and during or following steroid therapy in 5 patients (and in one patient who did not receive steroid therapy)[82]. Baseline PET imaging revealed intense pancreatic in all six patients. Intense ^{18}F-FDG uptake at pancreatic and extra-pancreatic sites resolved during or following steroid therapy in five patients; in the one other patient, who did not receive steroid therapy, pancreatic uptake resolved while uptake persisted at salivary glands and lymph nodes. In the series of Matsubayashi *et al*[69], extra-pancreatic uptake abnormalities were observed in 11 of 13 (85%) of cases; among 11 cases with follow-up PET imaging, abnormalities either resolved or decreased at three-month follow-up PET imaging in seven of nine (78%) cases.

The utility of FDG-PET in the staging and monitoring of IgG4-related disease was evaluated in a multicenter retrospective study involving 46 ^{18}F-FDG PET/CT examinations among 21 patients[83,84]. Imaging at diagnosis or onset of relapsed disease was available for 19 patients, with abnormal ^{18}F-FDG uptake detected among all 19 patients (100%). Results of FDG-PET/CT before and after treatment were available for 12 patients. Follow-up ^{18}F-FDG PET imaging demonstrated the following: complete normalization of ^{18}F-FDG uptake in five patients (42%); mixed response in three patients (25%), with sites of complete resolution, increase in uptake at existing sites, and foci of new uptake; no change in uptake abnormality in two patients (17%); and increased ^{18}F-FDG uptake despite treatment in two patients (17%), leading to new diagnoses of B-cell lymphoma and Castleman's disease. Correlative concurrent imaging *via* other modalities (US, CT, MRI) was available for 31 PET/CT evaluations. When abnormal findings from clinical examination or other imaging modalities were taken as the reference standard, the sensitivity for the PET/CT and CT to detect IgG4-RD organ involvement was 83% and 73%, respectively. False-negative PET/CT findings were associated with small focal lesions of the lacrimal glands, kidneys, lungs, and pachymeninges, or for inactive disease.

Given the multiple modalities available by which to diagnose and monitor the response to treatment in IgG4-related disease, further investigation correlating patient outcomes to imaging features, to assess for prognostic and predictive factors of treatment response and optimize patient care, are warranted.

CONCLUSION

Along with clinical, laboratory, and histopathological data, imaging plays an important role in the diagnosis and management of AIP, and more broadly, within the spectrum of IgG4-related disease. In addition to the defined role of imaging in consensus diagnostic protocols which have been established in order to discern AIP from important differential considerations such as pancreatic cancer, various imaging modalities can provide complementary data to address specific clinical concerns. These include contrast-enhanced CT and MR for pancreatic parenchymal lesion localization and characterization and ERCP and MRCP to assess for duct involvement. While the imaging appearance of AIP varies widely, certain imaging features are more likely to represent AIP than alternate diagnoses such as pancreatic cancer. Multiple systemic sites of involvement are often seen in AIP and IgG4-related disease, are amenable to CT, MR, and ^{18}F-FDG PET localization, and typically respond to corticosteroid therapy. Areas of further investigation include prognostic factors of treatment outcome, and optimal selection of imaging follow-up for treatment monitoring.

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2014 ADVANCES IN PANCREATITIS

Role of phosphoinositide 3-kinase in the pathogenesis of acute pancreatitis

Enrico Lupia, Luca Pigozzi, Alberto Goffi, Emilio Hirsch, Giuseppe Montrucchio

Enrico Lupia, Luca Pigozzi, Giuseppe Montrucchio, Department of Medical Sciences, University of Torino, 10126 Torino, Italy

Enrico Lupia, Luca Pigozzi, Emergency Medicine Unit, "Città della Salute e della Scienza" Hospital, 10126 Torino, Italy

Alberto Goffi, Interdepartmental Division of Critical Care Medicine, University of Toronto, Ontario ON M5G 1X5, Canada

Emilio Hirsch, Molecular Biotechnology Center, Department of Molecular Biotechnology and Health Sciences, University of Torino, 10126 Torino, Italy

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Correspondence to: Enrico Lupia, MD, Department of Medical Sciences, University of Torino, Via Genova 3, 10126 Torino, Italy. enrico.lupia@unito.it

Telephone: +39-11-6705395 Fax: +39-11-6705367

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Abstract

A large body of experimental and clinical data supports the notion that inflammation in acute pancreatitis has a crucial role in the pathogenesis of local and systemic damage and is a major determinant of clinical severity. Thus, research has recently focused on molecules that can regulate the inflammatory processes, such as phosphoinositide 3-kinases (PI3Ks), a family of lipid and protein kinases involved in intracellular signal transduction. Studies using genetic ablation or pharmacologic inhibitors of different PI3K isoforms, in particular the class I PI3Kδ and PI3Kγ, have contributed to a greater understanding of the roles of these kinases in the modulation of inflammatory and immune responses. Recent data suggest that PI3Ks are also involved in the pathogenesis of acute pancreatitis. Activation of the PI3K signaling pathway, and in particular of the class IB PI3Kγ isoform, has a significant role in those events which are necessary for the initiation of acute pancreatic injury, namely calcium signaling alteration, trypsinogen activation, and nuclear factor-κB transcription. Moreover, PI3Kγ is instrumental in modulating acinar cell apoptosis, and regulating local neutrophil infiltration and systemic inflammatory responses during the course of experimental acute pancreatitis. The availability of PI3K inhibitors selective for specific isoforms may provide new valuable therapeutic strategies to improve the clinical course of this disease. This article presents a brief summary of PI3K structure and function, and highlights recent advances that implicate PI3Ks in the pathogenesis of acute pancreatitis.

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Key words: Phosphoinositide 3-kinase; Cell signaling; Inflammation; Pathogenesis; Acute pancreatitis

Core tip: Phosphoinositide 3-kinases (PI3Ks) are a family of lipid and protein kinases implicated in intracellular signal transduction and regulation of inflammation. Recent data suggest their involvement also in the pathogenesis of acute pancreatitis. PI3Ks, and in particular the PI3Kγ isoform, have a significant role in those events which are necessary for the initiation of acute pancreatic injury, namely calcium signaling alteration, trypsinogen activation, and nuclear factor-κB transcription. Moreover, PI3Kγ modulates acinar cell apoptosis, and regulates local and systemic inflammatory responses during experimental acute pancreatitis. Specific PI3K inhibitors may therefore provide new therapies to improve the clinical course of this disease.

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INTRODUCTION

The process of pathologic autodigestion, triggered by prematurely activated digestive enzymes produced by acinar cells, has long been indicated as the key event for the initiation of acute pancreatic injury[1,2]. Recent research efforts have begun to clarify the biochemical mechanisms inducing intracellular zymogen activation[3-7], which include pathologic calcium signaling, alterations of intracellular trafficking that lead to the colocalization of lysosomal and zymogen-containing vacuoles, early activation of the nuclear factor-kappa B (NF-κB) pathway, autophagy, and oxidative stress[1-3]. Intracellular zymogen activation results in acinar cell necrosis and local inflammatory responses[8], which progressively resolve in most patients[9]. However, sustained inflammation may lead to the development of local and systemic complications and/or organ dysfunctions, which occur in about 20% of all cases of acute pancreatitis and account for the high mortality (10%-30%) of patients affected by severe acute pancreatitis[9-14].

A large body of experimental and clinical data supports the notion that inflammation in acute pancreatitis has a crucial role in the pathogenesis of local and systemic damage and represents a major determinant of clinical severity[9,15,16]. Increased levels of circulating inflammatory cytokines, chemokines and other humoral mediators have been reported in patients with acute pancreatitis[17,18], as well as in experimental *in vivo* and *ex vivo* (hyperstimulated acinar cells) models of the disease condition[3,6,14,19-22]. The molecular process underlying this event involves activation of specific transcription factors in the pancreatic tissue, including NF-κB, which is the most studied and best characterized of the transcription factors involved[6,22-27]. These humoral mediators, in turn, recruit neutrophils and then other immune cells from the bloodstream, such as macrophages, monocytes and lymphocytes, which amplify and sustain the inflammatory reaction in the pancreatic tissue[9,15,16]. Furthermore, experimental anti-inflammatory approaches - ranging from genetic deletion of cytokine receptors[28] or specific integrins[29,30], neutralization of cytokines, chemokines, adhesion molecules or other mediators[18,19,30-36], blockade of neutrophil recruitment[29,35-39], or complement inhibition[40] - have resulted in a significant reduction of mortality. However, whereas these experimental studies greatly improved our knowledge on the role of inflammation in the pathogenesis of acute pancreatitis, their results have not led to a progression in the treatment of patients affected by acute pancreatitis, and the few clinical trials conducted to date have yielded poor results[14,16,41-43]. Therefore, it is not surprising that research concerning the pathogenesis of acute pancreatitis has recently focused on the role of phosphoinositide 3-kinases (PI3Ks), a family of lipid and protein kinases involved in intracellular signal transduction and modulation of inflammatory and immune responses[44-48]. This article presents a brief summary of PI3K structure and function, with particular attention paid to their role in inflammatory pathologies, and discusses the recent advances involving PI3Ks in the pathogenesis of acute pancreatitis.

CLASSIFICATION AND STRUCTURE OF PI3KS

PI3Ks are a class of enzymes involved in intracellular signal transduction that were first described in the late 1980s[49,50]. They possess both protein and lipid kinase activity, with the latter function being the most extensively studied[45-47]. PI3Ks have historically been divided into three classes based on protein structure and substrate specificity[45-47].

Class I PI3Ks rely on the functional association of a catalytic subunit and a regulatory subunit, the latter of which modulates the activity of the heterodimer as well as its targeting to the plasma membrane upon receptor ligation, thereby allowing the enzyme access to the phosphatidylinositol substrates[45-47]. Class I PI3Ks have been further divided in two subgroups: IA and IB[45-47]. Class IA includes three members, PI3Kα, PI3Kβ and PI3Kδ, which are heterodimers composed by a specific p110 catalytic subunit (p110α, p110β and p110δ) and a regulatory p85 subunit. These isoforms are activated following stimulation of tyrosine kinase receptors, which include many growth factor receptors, such as those for epidermal growth factor[51], platelet-derived growth factor[52], fibroblast growth factor[53], growth hormone[54,55], insulin-like growth factor[56], insulin[57] and many interleukins (ILs)[58]. Nonetheless, a certain degree of isoform specificity has been demonstrated for several biological processes. For example, activation of the tyrosine kinase insulin receptor largely depends exclusively on PI3Kα[59,60]. On the contrary, PI3Kδ is specifically recruited in immune cells upon the activation of T and B cell receptors, natural killer stimulatory receptors, Fc receptors, and Toll-like receptors[61,62]. In addition, although class I PI3Ks usually act downstream of receptor tyrosine kinases, PI3Kβ is more effectively activated by G-protein-coupled receptors (GPCRs) than by tyrosine kinases[63-65]. PI3Kγ is the only member of the PI3K class IB, and its structural organization is represented by the association of either a p84/p87 or p101 regulatory subunit with the p110γ catalytic subunit[45-47]. PI3Kγ is activated by direct binding with G-protein βγ subunits, thus signaling downstream of GPCRs, such as chemokine receptors[45-47]. Moreover, PI3Kγ signaling activity can further be potentiated by Ras-GTP[66]. The main class I PI3K activity relies on the phosphorylation of phosphoinositides at the D3 position of the inositol ring, which leads to conversion of phosphatidylinositol (4,5)-bisphosphate to the second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$)[45-47,67,68]. PIP$_3$, upon membrane translocation, binds

with high affinity to the pleckstrin homology (PH) domain of its many effectors[45-47]. These effectors include protein kinases Akt/ protein kinase B (PKB), PDK1, Btk, GAP, and GEF for small GTPases, which mediate fundamental intracellular signaling events implicated in cell proliferation and migration, metabolic homoeostasis, and cell survival[45-47]. The signaling activity of class I PI3K is finely regulated by at least two lipid phosphatases, namely the SH2-containing inositol phosphatases 1 and 2 and the phosphatase and tensin homolog, which respectively dephosphorylate the inositol ring of PIP_3 on position 5 or 3[69-71].

The tissue distribution of class I PI3K isoforms is quite different: PI3Kα and PI3Kβ are widely expressed[45-47], whereas PI3Kγ and PI3Kδ are mainly expressed in leukocytes[44-47]. However, the expression of PI3Kγ has also been reported in the heart and in the endothelium[72,73], as well as in breast and pancreatic cancers[74-76]. Analogously, PI3Kδ expression has also been demonstrated in neurons, and in melanoma and breast cancer cells[77,78].

Class II PI3Ks are high molecular mass monomers, characteristically containing C2 and Phox homology (PX) domains that are fundamental for localization at the plasma membrane[45-47,79,80]. Their specific mechanism of activation and signaling, as well as their physiologic role in the regulation of cellular functions or their involvement in the pathogenesis of human diseases have only recently begun to be elucidated by the research[45-47,79,80]. For example, class II PI3K-C2α has been demonstrated as critically required for endocytosis[81] and for vascular integrity[82]. Interestingly, PI3K-C2γ is expressed in the exocrine pancreas[83], but its role in this organ remains largely unknown.

Finally, class III PI3K includes only one member, vacuolar protein sorting 34 (VSP34), which is only able to generate phosphatidylinositol 3-phosphate[45-47,80]. The physiologic importance of VSP34 and/or its involvement in human pathology are currently unclear[45-47,80].

Although very little is known about class II and III PI3Ks, there is increasing interest in developing inhibitors of these two classes for use as anticancer agents[45-48,79,80].

ROLE OF PI3KS IN INFLAMMATORY CELLULAR RESPONSES

The involvement of PI3Ks in inflammation has been recently highlighted by studies using genetic or pharmacologic inhibition of different PI3K isoforms[45-47]. Genetic ablation of PI3Kα and PI3Kβ was lethal during embryonic development[84,85]; however, PI3Kδ and PI3Kγ knock-out mice were viable and mainly showed alterations of both innate and adaptive immune responses[86-89]. Ultimately, those results led to a better characterization of the regulatory role of these two PI3K isoforms in inflammatory pathologies.

PI3Kγ and PI3Kδ act in partnership to regulate the recruitment of neutrophils and monocyte/macrophages to the site of inflammation and then to coordinate the respiratory burst[44-47]. In PI3Kγ-null mice, neutrophils and macrophages display reduced migration in response to different stimuli that act through GPCRs, such as N-formylated peptides (fMLPs), C5a, or IL-8[72,86-88]. In addition, *in vivo* investigation of a peritonitis mouse model showed highly impaired leukocyte recruitment[86-88]. On the contrary, PI3Kδ appears to be specifically involved in regulating the directional neutrophil movement in response to chemotactic agents[90,91]. Endothelial activity of both PI3Kγ and PI3Kδ also has a role in regulating neutrophil adhesion to inflamed vessel wall[91,92]. At the inflammatory sites, PI3Kγ and PI3Kδ also cooperate in order to regulate the production of reactive oxygen species; this is a biphasic process in which the initial phase is dependent on PI3Kγ activation and is followed by an amplification phase mediated by PI3Kδ[86-88,90,91].

In addition to the roles of PI3Ks in neutrophils and monocytes, these kinases also regulate fundamental cellular functions in mast cells and eosinophils[45-47]. Pharmacological inhibition of PI3Kδ reduces degranulation and cytokine release induced in mast cells by immunoglobulin (Ig)E stimulation[93,94] and protects mice from passive cutaneous anaphylaxis induced by IgE and antigen injection[93,94]. In addition, inhibition of PI3Kγ decreases adenosine-induced mast cell degranulation and resistance to passive systemic anaphylaxis[95], demonstrating a specific role for this kinase in sustaining and maximizing mast cell degranulation[93,95]. Furthermore, PI3Kγ is involved in eosinophil recruitment, modulation of allergen-induced eosinophilic airway inflammation, and airway remodeling[96,97].

PI3K activity is also involved in regulation of the cellular functions of T and B lymphocytes, the main actors of the adaptive immune response[45-47]. Both PI3Kγ and PI3Kδ are considered crucial for T cell development[45-47], since knock-out mice for either one or the other kinase show reduced numbers of peripheral T lymphocytes and increased ratios of double-negative ($CD4^-CD8^-$) to double positive ($CD4^+CD8^+$) cells in the thymus[87,98,99]. Moreover, PI3Kδ is heavily involved in $CD4^+$ T cell maturation and differentiation in distinct T cell subsets[45-47,61], whereas PI3Kγ is involved in T cell receptor-stimulated proliferation and cytokine production[61,87]. PI3Kδ is also involved in the regulation of B cell maturation and activation[45-47]. PI3Kδ-null mice showed an increased proB/preB ratio, which was due to a blockade of the maturation process that occurs between these two stages[89,100,101], as well as reduced IgM and IgG antibody responses, which were associated with a paradoxical increase in production of IgE[89,102,103]. In line with these critical functions, PI3Kδ and PI3Kγ/δ inhibitors show important anti-proliferative activity in different forms of human hematologic malignancies, with particular efficacy in lymphomas[104].

PI3KS IN INFLAMMATORY DISEASES

PI3Kγ and PI3Kδ have been extensively investigated as

potential therapeutic targets in autoimmune and allergic diseases, and in pathologic conditions where inflammation has a crucial role for onset and progression[44-48].

Blockade of PI3Kγ by genetic ablation or by using selective pharmacological inhibitors reduces the incidence and severity of disease in the MRL-lpr mouse model of systemic lupus erythematous[105] and in two different experimental models of rheumatoid arthritis, induced either by collagen injection or by transgenic overexpression of human tumor necrosis factor-α[106,107]. Inhibition of PI3Kδ also reduces inflammation and bone and cartilage erosion in a model of arthritis induced by the administration of arthritogenic serum[108].

Consistent with the role of PI3Ks in mast cell and eosinophil activation[93-97], genetic ablation of PI3Kγ reduces leukocyte infiltration, hyper-responsiveness, and airway remodeling in an ovalbumin (OVA)-induced model of asthma[96,97,109]. Similarly, inhibition of PI3Kδ either by genetic ablation or specific inhibitors decreases eosinophil infiltration, T helper cell (Th2) cytokine production (IL-4, IL-5 and IL-13), bronchiolar inflammation, and airway remodeling in the same OVA-induced asthma model[110,111].

PI3Ks are also involved in the pathogenesis of cardiovascular diseases in which inflammation has a relevant role, namely atherosclerosis and myocardial infarction[46]. PI3Kγ inhibition is effective in reducing plaque size in a model of early-stage atherosclerosis (apolipoprotein E-null mice)[112] and in the more aggressive low-density lipoprotein receptor knockout (LDLR$^{-/-}$) model that mimics progressive familial hypercholesterolemia[113]. Interestingly, transplantation of bone marrow from PI3Kγ-null mice into LDLR$^{-/-}$ mice also reduces plaque size[113], indicating that the formation of atherosclerotic lesions is regulated by PI3Kγ expressed by immune cells. Moreover, PI3Kγ inhibition has been found to influence cellular composition of atherosclerotic plaques (as suggested by the observation of a reduction of infiltrating macrophages and T cells) and to increase plaque stability[113]. Finally, in agreement with the pathogenic role of inflammation in ischemia-reperfusion injury, TG100-115, a dual inhibitor of PI3Kγ and PI3Kδ, reduces infarct size and preserves myocardial function in an *in vivo* model of myocardial infarction[114].

PI3KS IN ACUTE PANCREATITIS

Little is known about the physiological role of PI3Ks in pancreatic acinar cells[115]. However, pharmacologic analysis has implicated PI3Ks in cholecystokinin (CCK)-induced phosphorylation of p70S6 kinase and focal adhesion kinase and in regulation of exocytosis[115-118].

The involvement of PI3Ks in the pathogenesis of acute pancreatitis was first demonstrated in a study by Singh *et al*[119] using two unrelated inhibitors of all PI3K isoforms, wortmannin and LY294002, in two different rodent models of acute pancreatitis, one induced by supramaximal secretagogue stimulation and the other by duct

injection. In the cerulein-induced model, wortmannin administration inhibited early trypsinogen activation, an effect associated with reduced redistribution of cathepsin B and intracellular colocalization of lysosomal hydrolases with digestive enzyme zymogens[119]. Moreover, wortmannin reduced the extent of pancreatic edema, neutrophil sequestration within the pancreas, acinar cell necrosis, and hyperamylasemia in the same model. Wortmannin also reduced pancreatic trypsin activity, acinar cell necrosis and myeloperoxidase activity in the second acute pancreatitis model, which had been induced by retrograde infusion of the rat pancreatic duct with the bile salt sodium taurocholate. *Ex vivo* experiments showed that wortmannin and LY294002 inhibited cerulein-induced trypsinogen activation without affecting the changes to the cytoskeleton of acinar cells that had been induced by supramaximal cerulein stimulation, in particular the redistribution of F-actin from subapical to basolateral areas[119]. The authors also performed experiments aimed to identify which class of PI3K was involved in trypsinogen activation during pancreatitis, initially directed toward class I PI3K because of its known association with GPCRs, such as CCK receptors. However, supramaximal concentrations of cerulein, those that induced *ex vivo* trypsinogen activation, did not increase phosphatidylinositol-3,4-bisphosphate nor PIP3, nor did they induce phosphorylation of Akt/PKB in these experiments[119], suggesting that class I PI3K were not involved. These results differ from those previously reported by another group, which had shown formation of class I PI3K products after stimulation with maximal concentrations of cerulean[120]. On the contrary, both in unstimulated and cerulein-stimulated acini, wortmannin decreased levels of the product of class III PI3K, phosphatidylinositol 3-phosphate, which is implicated in vesicle trafficking and fusion[119]. The authors proposed that cerulein-induced intra-cellular trypsinogen activation may be a consequence of perturbed vesicle trafficking induced by the accumulation of the phosphatidylinositol 3-phosphate class III PI3K product in a yet unidentified subcellular compartment[119].

Subsequent studies by different research groups have further analyzed the specific role of the PI3Kγ isoform in the pathogenesis of acute pancreatitis. Gukovsky *et al*[121] used PI3Kγ-deficient mice as well as pharmacologic PI3K inhibitors to investigate the role of PI3K in CCK-induced responses in isolated pancreatic acinar cells. These experiments showed that both PI3Kγ genetic ablation and PI3K inhibition greatly diminished the CCK-induced calcium response in pancreatic acini by inhibiting both intracellular calcium mobilization and calcium influx, showing that PI3Kγ is required for pathologic calcium responses to CCK hyperstimulation[121]. Further studies by the same group demonstrated that PI3Kγ regulates calcium signaling in pancreatic acinar cells by inhibiting sarco(endo)plasmic reticulum calcium-ATPase[122,123]. In addition to its regulatory role on calcium signaling, PI3Kγ is also implicated in regulating trypsinogen activation[121]. CCK-induced trypsinogen activation was, indeed, reduced

by about 60% in pancreatic acini isolated from PI3Kγ-null mice[121], an effect that may also be partially mediated through calcium signaling[121]. Finally, both PI3K inhibitors and PI3Kγ genetic deletion inhibited CCK-induced NF-κB activation *in vitro*, indicating a regulatory role for PI3Kγ in the NF-κB response[121]. This result did not confirm those previously reported by Singh *et al*[119] in the rat cerulein-induced pancreatitis model; however, in that study, NF-κB activation was only measured at one time point and only *in vivo*, not *ex vivo*[119]. Of note, CCK-elicited responses in PI3Kγ-null isolated acini were further inhibited by LY294002, implicating involvement of other PI3K isoforms[121].

Our research group independently studied the effects of genetic ablation of PI3Kγ on the severity of acute pancreatic damage induced *in vivo* by supramaximally stimulating doses of cerulein or administration of a choline-deficient, ethionine-supplemented (CDE) diet[124]. Although amylase secretion in isolated pancreatic acini was not different in PI3Kγ-null mice compared to wild-type mice, the genetic ablation had significantly reduced the extent of acinar cell injury/necrosis in both models. A partial but significant reduction in the extent of acinar cell injury/necrosis was evident six hours after the beginning of cerulein administration. On the contrary, serum amylase levels were not decreased and pancreatic water content was even increased in the PI3Kγ-deficient mice compared to the wild-type mice. In addition, only minimal neutrophil infiltration was seen at time points as early as six hours. Therefore, this protective effect can likely be ascribed to the lack of PI3Kγ influence on the early intra-acinar cell events, as indicated elsewhere[121]. Our study also showed an increase in the number of apoptotic acinar cells in PI3Kγ-null mice (identified by terminal dUTP nick-end labeling and caspase-3 activity), which is consistent with the described protective role of apoptosis in acute pancreatitis[125,126]. As we did not observe any activation of Akt/PKB, the major effector of PI3K survival signaling[127,128], it can be hypothesized that PI3Kγ may interfere with other death signaling pathways, such as caspase activation, cytochrome c release, or mitochondrial depolarization, which have been implicated in the direct pro-apoptotic effect exerted by supramaximal concentrations of CCK in pancreatic acini[129].

We also observed a significant reduction of both acinar cell injury/necrosis and neutrophil infiltration in PI3Kγ-null mice after prolonged administration of cerulein for 13 h[124]. This protective effect may be related to the ability of PI3Kγ to regulate the neutrophil chemotaxis and respiratory burst that follows neutrophil activation[44-48] or to enhance neutrophil apoptosis, thus favoring the removal of activated neutrophils from the pancreatic tissue[130]. Moreover, cerulein-induced pancreatic COX-2 up-regulation, which modulates the course of acute pancreatitis[131-133], was also blunted in the PI3Kγ-null mice, likely contributing to the observed protective effect of genetic ablation[124].

PI3Kγ deletion was also found to reduce acinar cell injury/necrosis, neutrophil infiltration and lung injury in a second model of necrotizing acute pancreatitis induced by administration of a CDE diet[124]. Furthermore, the genetic ablation reduced the mortality rate, indicating that PI3Kγ influences the development of injury to other organs, in particular the lungs. Indeed, a recent study by another group has shown that the PI3K-Akt pathway mediates the protective effect exerted by estrogens on lung injury during cerulein-induced acute pancreatitis[134], indirectly confirming our hypothesis.

PI3Kγ is also known to possess scaffold functions that regulate cAMP levels[72,135], and it can bind protein kinase A (PKA) and different phosphodiesterases[136] to control a PKA-mediated negative feedback signal that promotes cAMP destruction. Given the importance of cAMP elevation in the protection from acute pancreatitis[137], it is therefore possible that some of the effects of PI3Kγ are independent of its catalytic activity.

CONCLUSION

The activation of PI3Ks, and in particular of the class IB PI3Kγ isoform, has a relevant role in the biochemical events, namely calcium signaling alteration, trypsinogen activation, and NF-κB transcription, all of which are necessary for the initiation of acute pancreatic injury. The ability of PI3Kγ to modulate acinar cell apoptosis, as well as to regulate local neutrophil infiltration and systemic inflammatory responses during the course of acute pancreatitis, renders PI3Kγ an ideal therapeutic target. The availability of inhibitors selective for specific PI3K isoforms might provide new valuable therapeutic strategies to improve the clinical course of this disease.

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2014 ADVANCES IN PANCREATITIS

Immune-modulating therapy in acute pancreatitis: Fact or fiction

Karolina Akinosoglou, Charalambos Gogos

Karolina Akinosoglou, Charalambos Gogos, Department of Internal Medicine and Infectious Diseases, University General Hospital of Patras, 26504 Rio, Greece
Author contributions: Akinosoglou K and Gogos C performed literature searches and reviewed appropriate articles; Akinosoglou K wrote the manuscript and drew figures; Gogos C critically corrected the manuscript.
Correspondence to: Karolina Akinosoglou, MD, Department of Internal Medicine and Infectious Diseases, University General Hospital of Patras, 5th Floor, 26504 Rio, Greece. akin@upatras.gr
Telephone: +30-2610-999582 **Fax:** +30-2610-999582
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Core tip: Acute pancreatitis is a common entity with significant mortality worldwide. Treatment remains non-specific and mainly supportive, mostly focusing on intensive care. Presence of inflammatory response syndrome during AP has driven recent immune-modulating therapeutic attempts in experimental models, including cytokine, chemokine, immune cell and other inflammatory mediator blockade. Although initial data are promising, translation to clinical routine has been less encouraging. The authors attempt to elucidate whether and to what extent tampering with the immune burst triggered by acute pancreatitis could actually ensure better outcomes, or that remains a farfetched expectation.

Abstract

Acute pancreatitis (AP) is one of the most common diseases of the gastrointestinal tract, bearing significant morbidity and mortality worldwide. Current treatment of AP remains unspecific and supportive and is mainly targeted to aggressively prevent systemic complications and organ failure by intensive care. As acute pancreatitis shares an indistinguishable profile of inflammation with sepsis, therapeutic approaches have turned towards modulating the systemic inflammatory response. Targets, among others, have included pro- and anti-inflammatory modulators, cytokines, chemokines, immune cells, adhesive molecules and platelets. Even though, initial results in experimental models have been encouraging, clinical implementation of immune-regulating therapies in acute pancreatitis has had a slow progress. Main reasons include difficulty in clinical translation of experimental data, poor understanding of inflammatory response time-course, flaws in experimental designs, need for multimodal approaches and commercial drawbacks. Whether immune-modulation in acute pancreatitis remains a fact or just fiction remains to be seen in the future.

INTRODUCTION

Acute pancreatitis (AP) is a common disease, posing a tremendous burden in health care systems globally[1,2]. The incidence of AP varies between 4.9 and 73.4 cases per 100000 worldwide[3,4]. Progression to multiple organ dysfunction syndrome (MODS), as a consequence of the systemic inflammatory response syndrome (SIRS) represents a major contributor to high mortality in the early phase of the disease[5,6]. Consequent breakdown of
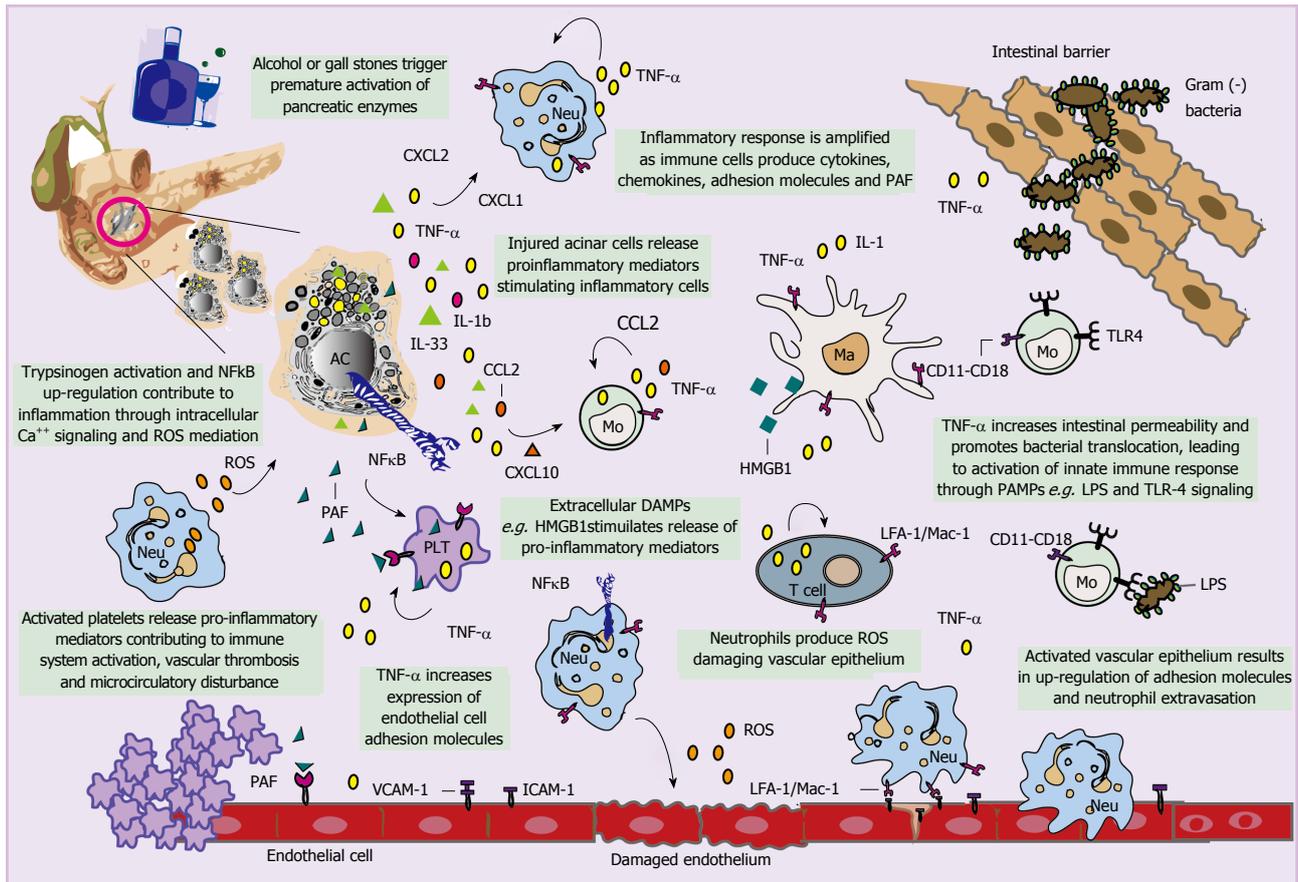
Figure 1 Schematic representation of innate and adaptive immune response mechanisms implicated in acute pancreatitis. Triggering factors initiate trypsinogen activation and pancreas autophagy. Damaged acinar cells release damage-associated molecular pattern molecules (DAMPs) and pro-inflammatory mediators attracting leukocytes at the site of inflammation. Leukocyte activation leads to increased leukocyte aggregation through increased expression of adhesion molecules and tissue infiltration within the microcirculation. There, these cells increase production of cytokines and other inflammatory mediators including prostaglandins, leukotrienes, thromboxanes, platelet activating factor (PAF), free radicals, nitric oxide and proteases. These substrates increase vascular permeability resulting in neutrophil extravasation and activation, oedema and microvascular disturbances which eventually lead to lack of oxygen and tissue injury. Pro-inflammatory mediators contribute to failure of intestinal barrier function and translocation of intestinal microflora or their products into the vascular bed. Neu: Neutrophil; AC: Acinar cell; Mo: Monocyte; Ma: Macrophage; ICAM-1: intercellular adhesion molecule 1; ROS: Reactive oxygen species; VCAM-1: Vascular adhesion molecule 1; TNF-α: Tumour necrosis factor alpha; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; IL: Interleukin; CXCL: C-X-C motif chemokine; CCL: Chemokine (C-C motif) ligand; PLT: Platelet; TLR: Toll like receptor; LPS: Lipopolysaccharide; LFA: Lymphocyte function associated antigen; Mac-1: Macrophage-1 antigen.

intestinal integrity, bacterial translocation and increased infection risk can further complicate outcome in the late phases of AP[7-9].

Current treatment of AP remains non-specific and supportive and is mainly targeted to aggressively prevent systemic complications by intensive care. During the last decade, a number of new therapeutic modalities have changed the management of acute pancreatitis, including enteral feeding in severe AP, the use of early antibiotic treatment in necrotizing pancreatitis and therapeutic endoscopic retrograde cholangiopancreatography (ERCP) with endoscopic sphincterotomy in severe biliary pancreatitis[10,11]. However, although the case fatality rate for AP has decreased over time, the overall population mortality rate has remained unchanged[12].

Recent data has come to show that in the early phase of AP, excessive leukocyte activation and consequent inflammatory mediator release are critical for development of early organ failure[13-16]. As a result, current experimental and clinical research has been driven by the need to inhibit the systemic inflammatory reaction thus; prevent the development of MODS. This article attempts to critically review recent data on immune-modulating strategies in AP.

LOCAL AND SYSTEMIC INFLAMMATION

Pancreatic self-digestion

Acute pancreatitis represents an inflammatory disorder; hence, a complex cascade of immunologic events affects disease pathogenesis and progression. Alcohol and gallstones remain the major etiologic factors of AP. Irrespective of the cause, triggering events lead to premature activation of pancreatic proteases, as a result of intracellular co-localization with lysosomal enzymes[17,18]. An increase in intracellular calcium triggers activation of trypsinogen and induces local inflammation[19], further leading to auto-digestion, destruction of the parenchyma and finally necrosis of the pancreas[17,20-23](Figure 1).

The role of tumour necrosis factor alpha (TNF-α)

in the potential activation of pancreas polypeptide and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[24] has been recently investigated[25-29]. Whether there is a link between pancreatic NF-κB and trypsinogen activation remains controversial[27]. However, it seems that these processes may be unrelated and may both contribute to inflammation, possible through reactive O_2 species (ROS) mediation and calcium (Ca^{2+}) signalling[30,31] (Figure 1).

At this stage, it appears that the autophagy machinery interfaces with various cellular stress-response pathways including those involved in immune response and inflammation, entailing among others direct interactions between autophagy proteins and immune signalling molecules[32-34]. This complex interplay modulates both the induction and suppression of immune and inflammatory responses and *vice versa*, while it seems that the same genes that regulate autophagy are involved in xenophagy[35,36]. In this respect, a potential protective role for interleukin (IL)-22 against the autophagic pathway in pancreatitis is currently under investigation[37].

Leukocyte activation and cascade reaction

Endogenous molecules released as a result of tissue injury, referred to as damage-associated molecular patterns represent primary activators of the immune system[38-40]. Among those, high mobility group box 1 (HMGB1) protein, a nuclear DNA binding protein[41-43], has been recently suggested to act as a key mediator for inflammation and organ failure in AP[44-46]. Pancreatic-derived intracellular HMGB1 limits the severity of the disease by protecting cells from NF-κB activation, DNA damage, cell death, and release of nucleosomes from injured acinar cells[47]. On the other hand, extracellular HMGB1 released by necrotic cells, can, *via* members of the Toll-like receptor (TLR) family trigger acute lung injury[48,49] and a lethal systemic inflammatory process[50,51]. Extracellular HMGB1 can further stimulate the release of pro-inflammatory cytokines including TNF-α and IL-1β by inducing nuclear translocation of NF-κB and conversely, the pro-inflammatory cytokines can control further release of HMGB1 into the extracellular space (Figure 1)[52-54].

Activated acinar cells also secrete pro-inflammatory factors including C-X-C motif chemokine (CXCL) 10, Chemokine (C-C motif) ligand 2 also referred to as monocyte chemotactic protein-1 (MCP-1), IL33[55,56], platelet activating factor (PAF), TNF-α and IL-1β leading to migration of monocytes and neutrophils into the pancreas[57,58]. Neutrophils are specifically activated by CXCL-1 and CXCL-2 (also called macrophage inflammatory protein 2-alpha, MIP2-α), while monocytes, eosinophils and T-cells are activated by CCL-2 (MCP-1) and CXCL-10[59] (Figure 1). However, monocyte and macrophage populations involved in AP are heterogeneous, with great phenotypic and functional plasticity[60]. Recently, a subtype of monocytes that derive from the bone marrow and express TNF-α has been identified, which appears to determine pancreatic oedema and acinar cell

injury/necrosis[61]. T cells are also present in smaller numbers in the inflamed pancreas and appear to be necessary for progression of AP[62]. As AP progresses, changes in the number and ratio of CD4+ and CD8+ T cells has been noted, probably because CD4+ T cells contribute to activation of macrophage *via* antigen presentation and release of inflammatory cytokines[63]. In contrast to total depletion of CD4+ T cells, and consistent with functional heterogeneity of CD4+ T cells, recent data indicate that a subset of CD4+ IL22+ T cells likely protects against AP in mice, even though exact mechanisms remain elusive[64].

The magnitude of the inflammatory process is amplified following further secretion of inflammatory mediators by infiltrating immune-associated cells[65-67], and over-expression of adhesion molecules including intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1[68,69].The latter represent ligands for lymphocyte function-associated antigen 1[70] on leukocytes and lymphocytes, αLβ2 and CD11a-CD18 on monocytes and integrin macrophage 1 antigen (Mac-1) on neutrophils, while their secretion is promoted by ROS generation and TNF-α itself (Figure 1)[71-73]. Notably, ICAM-1 deficiency and systemic depletion of neutrophils were each shown to reduce the severity of AP and lung injury[71].

Bacterial translocation

Except for regulation of cellular apoptosis, TNF-α was shown to increase intestinal paracellular permeability, by affecting tight junctions[74] and facilitating bacterial translocation from the epithelium[75]. It has been suggested that, pathogen-associated molecular patterns derived from the intestinal micro flora activate the host innate immune system *via* pattern recognition receptors, such as TLRs and nucleotide-binding domain and leucine-rich repeat-containing molecules[76] (Figure 1). Activation of TLRs and nucleotide-binding domain and leucine rich repeat-containing molecules likely mediates the mechanism by which bacterial translocation leads to severe AP. Consistent with this, mice that lack TLR4 develop less severe forms of AP[77], and polymorphisms in *TLR* genes have been associated with susceptibility to AP[78,79]. Interestingly, up-regulation of TLR4 has been associated with increased expression of TNF-α in peripheral blood mononuclear cells during early stages of AP[80].

Pancreatic microcirculatory disturbance

Various molecules and mechanisms appear to complete the full spectra of manifestations in AP, mainly attributed to microcirculatory disturbance including nitric oxide, endothelin, oxygen free radicals, bradykinin, prostaglandin I2 and endothelin[81]. Inflammatory mediators induce microcirculatory disturbance mainly through increasing capillary permeability and decreasing capillary blood flow velocity (such as ICAM-1), promoting the contraction of arteries and veins (such as endothelin), as well as, promoting platelet aggregation and inducing thrombosis (such as PAF and TXA2). In the latter case, PAF exerts its biologi-
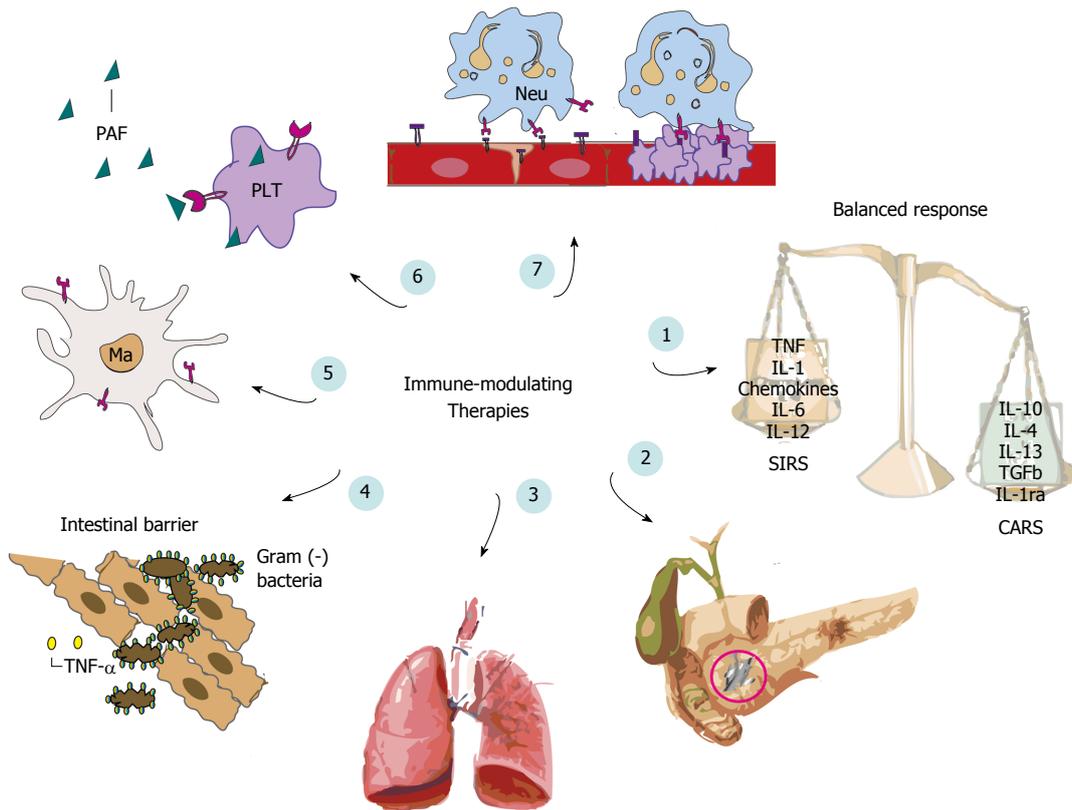
Figure 2 Immune modulating therapies. (1) Target inflammatory response promoting or attenuating anti and pro-inflammatory mediators respectively (recombinant cytokine/chemokine administration, cytokine/chemokine antagonists, receptor blockade); (2) ameliorate parenchymal and fatty tissue necrosis of the pancreas [*e.g.*, infliximab, blockade of interleukin (IL)-1 receptor, IL-10 blockade, IL-12 suppression, platelet activating factor (PAF)]; (3) alleviate alveolar oedema and development of acute respiratory distress syndrome (*e.g.*, infliximab, IL-10 blockade, IL-8 blockade); (4) correct intestinal barrier and prevent bacterial translocation (*e.g.*, anti-tumour necrosis factor, TNF); (5) modulate immune cell response (*e.g.*, stem cell immunosuppressive strategies); (6) impair platelet activation and further immune activation (*e.g.*, PAF); and (7) protect against endothelial barrier dysfunction, transmigration of neutrophils and concomitant microcirculatory derangements (*e.g.*, adhesion molecule blockade). Ma: Macrophage.

cal activity through binding to its specific receptors on the surface of leukocytes, endothelial cells and platelets leading to microcirculatory disturbance in AP[82-85] (Figure 1). Furthermore, an increasing body of evidence reveals a pro-inflammatory role of platelets except for their established function in thrombosis and haemostasis[86-88]. During AP, data have come to show that platelets regulate neutrophil accumulation in the pancreatic tissue[89], even though exact mechanisms underlying platelet dependent leukocyte recruitment remain elusive. At the moment these mechanisms and various molecules[90], although important; surpass the purpose of this review and will not be discussed further. Figure 1 represents a schematic summary of innate and adaptive immune response mechanisms implicated in acute pancreatitis.

In mild AP, inflammation is regulated and confined by the host's inflammatory response in the affected area. Although, most episodes of AP are mild, some patients proceed to SIRS, as a result of pro-inflammatory mediators' release into the circulation[91,92], with local and extra-pancreatic complications[93], including respiratory, renal and hepatic dysfunction[94,95]. Systemic inflammation in AP is concomitantly associated with rapidly strengthening compensatory anti-inflammatory response syndrome

(CARS)[96]. Even though, CARS may be sufficient to control SIRS and ensure favourable prognosis, excessive CARS may be overwhelming, leading to immune deficiency or suppression, which renders the host susceptible to secondary infections[97]. Increased serum concentrations of anti-inflammatory mediators including IL-10, IL-11, TNF-α receptors, and IL-1 receptors antagonist (IL-1ra) have been demonstrated in AP[98-102]. In immune-suppression monocytes are characterized by a significantly decreased human leukocyte antigen antigen-DR (HLA-DR) expression, process mostly attributed to IL-10 production[103]. Along with consequent impaired antigen presentation[104,105], monocytes show a profound reduction of their ability to produce pro-inflammatory cytokines *e.g.*, TNF-α[106,107], facts associated with development of secondary infections[97] and organ failure[108,109]. IL-1ra and IL-6 are also important anti-inflammatory cytokines. IL1-ra blocks IL-1 mediated responses[110] while IL-6 appears to prevent synthesis of IL-1β and TNF-α[111].

THE STORY OF IMMUNE-MODULATING THERAPIES: FACT…

Following understanding that, outcomes of our patients

seem to be mostly dependent on pro- as well as, anti-inflammatory responses; in the last few years, a number of experimental and clinical studies have focused their interest in immune regulation during AP (Figure 2).

TNF-α

Recent data in animal models has come to show that, TNF-antagonism by either TNF-receptor blockade or anti TNF-antibodies protected from local intra-pancreatic damage, systemic complications and overall mortality in the vast majority of cases[29,112-117]. Administration of infliximab -a monoclonal TNF-antibody- appears to decrease serum amylase activity in both acute oedematous pancreatitis and severe necrotizing pancreatitis in a murine model[118]. In the latter case, a tendency to ameliorate both parenchymal and fatty tissue necrosis of the pancreas and alleviate acute respiratory distress syndrome-like pulmonary complications, was also noted. However, even though TNF-α has been clearly associated with failure of intestinal barrier, the latter study provided no information upon infliximab's role on intestinal permeability since bacterial translocation was not considered a septic complication. However, previous reports that TNF-α blockage appears to correct intestinal permeability in people with Crohn's disease[119], suggests that this effect may occur in other types of disorders and could represent a feasible therapeutic option. In a recent study, Aydin *et al*[120] showed that, infliximab administration 6 h after the induction of pancreatitis exerted beneficial effects on blood amylase levels and histopathologic changes in experimental necrotizing pancreatitis, while significant decrease in the degree of BT was noted. Even though, premature administration of anti-TNF 6 h post pancreatitis induction was beneficial, previous reports support that a prophylactic design starting the treatment before the induction could be superior, while the protective effect of TNF-antagonism on disease severity and mortality was still observed after the systemic effects had developed[116]. To date, no study has been conducted investigating TNF-a impact in clinical acute pancreatitis except for an isolated report in a patient with Crohn's disease complicated with acute pancreatitis[121]. Interestingly, favourable outcome was observed following infliximab's administration. However, data from two phase Ⅲ sepsis trials[122,123] sharing an indistinguishable profile of inflammatory mediators with AP, have not been that optimistic. The use of an anti-TNF antibody in patients with sepsis failed to reduce 28-d mortality, suggesting that previous results from experimental studies or anecdotal reports should be interpreted with caution.

Cytokines

As previously described, pro-inflammatory cytokines such as IL-6, IL-1 and TNF are released in acute pancreatitis, while their plasma level correlates well with the severity of the disease and the occurrence of multi-organ failure[124-126]. By contrast, anti-inflammatory mediators such as IL-10, appear to mitigate the effects of inflammatory response and their level seems to be inversely proportional to the severity of pancreatitis[127,128].

IL-1: As observed for TNF, organ-specific expression of IL-1 is an early feature in experimental acute pancreatitis and is found in both the pancreas and distant organs[129,130]. Blockade of the IL-1 receptor by either targeted genetic disruption or pharmacological agents uniformly reduced the extent of intra-pancreatic damage, systemic complication, and mortality, similarly to TNF-α blockade[131-135]. Alternatively, the approach of inhibiting caspase-1, formerly termed interleukin 1β-converting enzyme (ICE) has been explored. Targeting ICE activity by a specific synthetic inhibitor dramatically attenuated both severity and mortality irrespective of the model used[136-139]. Interestingly, severity and mortality were still reduced even when a therapeutic window of 12 h following induction of severe acute pancreatitis was allowed[137]. Similarly to TNF-α, implementation of experimental findings to the clinical setting has been controversial. Even though, a post hoc analysis of a controlled trial of human recombinant IL-1ra in patients with sepsis showed a trend towards increased survival in patients with MODS[140], this observation could not be confirmed in a subsequent trial[141]. Technical reasons concerning the optimal dosage, duration and *in vivo* activity of the antibody could be responsible for these discrepancies[142].

IL-10: IL-10 - irrespectively of whether its activity has been blocked or augmented -has been shown to exert a protective effect in several models of acute pancreatitis in the past[143-149]. Cytokine manipulation appeared to significantly ameliorate organ specific damage in the pancreas and peripheral tissues, including the lung and the liver, while mortality was significantly reduced. Interestingly, IL-10 protective effect was still observed even when intervention occurred therapeutically after acute pancreatitis had already been induced[144,145,149]. However, this data has not been confirmed in the clinical setting. No significant differences were detected between IL-10 and placebo administration within 36 h of onset of symptoms, in days of hospital stay, CT scan score, organ failure score and local complications[150]. Conflicting results have come to light from randomized double-blind studies regarding the ability of IL-10 to prevent ERCP–induced AP. In his study, Devière *et al*[149] reported that, IL-10 decreased the incidence of post-ERCP pancreatitis, as well as, the length of hospital stay independently from other risk factors. This was not confirmed by a later American trial in which only a trend towards and not a significant decrease in the former parameters was noted[151]. A recent meta-analysis including patients receiving recombinant IL-10 or placebo before ERCP could show that, IL-10 significantly reduces the risk of post-ERCP pancreatitis[152]. Whether IL-10 treatment can ultimately prevent post-ERCP still remains under investigation.

IL-2: In contrast to the late effects of IL-2 deficiency

and immune-paralysis, the excessive IL-2 mediated T-cell response during the early course of the disease can be deleterious[62,153-157] Following transcriptional regulation by administration of the FK506 agent which inhibits IL-2 production, decreased early local and systemic disease severity was noted even when given in a therapeutic fashion[155]. Similarly, early data have come to show that, sirolimus, an immune-modulatory agent, acting through inhibition of response to IL-2, thereby blocking activation of T and B cells, reduces acute pancreatic damage in the first week and less chronic changes in the further course of disease[157]. However, opposite results were shown by other studies. FK506 administration significantly worsened survival in diet-induced murine pancreatitis[156] while potentiation of IL-2 production through levamisole administration effectively decreased the incidence of pancreatic infections in a cat model of severe acute pancreatitis[153].

IL-18: IL-18 or interferon-inducing factor represents a novel key regulator of Th-1 response, through its ability to induce IFN-production in T and natural killer cells[158]. Scarce data on this interesting cytokine have shown that intra-pancreatic damage was decreased more effectively following neutralization of IL-18 activity by monoclonal antibodies than neutralizing IL-1 activity in cerulein-induced pancreatitis in mice[159]. Further research in IL-18 and its role during AP is currently in progress.

IL-6: Despite the numerous clinical studies investigating the role of IL-6 in the development of inflammatory syndrome, unfortunately only few reports have examined the role of this interleukin as potential target for modulating disease severity. Even though, IL-6 has been found to be a good predictor in predicting pancreatitis associated complications, including organ failure[160], limited experimental data have revealed that genetic deletion or prophylactic inhibition of IL-6 rather worsened outcomes than exerted a protective effect on disease severity and mortality[161-163].

PAF

Following previous observations implicating PAF in classical morphologic and biochemical derangements induced during AP[164], a number of groups have pursued the potential therapeutic role of this novel cytokine[165-171]. Except for one case[172], pathophysiological changes of acute pancreatitis were reduced in all established experimental models following PAF antagonism[168-171]. When PAF antagonists were applied therapeutically, local intra-pancreatic damage and micro-circulatory derangement was significantly ameliorated while systemic complications and mortality was considerably decreased[168,170,171]. One of the most promising PAF antagonists Lexipafant has been recently tested in two phase Ⅱ trials including patients with acute[173] or predicted severe pancreatitis[174]. Results were encouraging, showing significant improvement of organ failure or organ failure scores. Subsequently though, in a

randomized, double blind trial encountering patients with severe AP, intravenous administration of lexipafant for 7 d did not show any benefit in reducing MODS or mortality[175]. Nonetheless, systemic levels of IL-8 and E-selectin, sepsis or pseudocysts development were significantly lower in the treated than in the non-treated group, especially in patients treated within 48 h from the onset of symptoms[175]. In view of the multiple prior experimental studies suggesting that lexipafant is highly effective in reducing SIRS associated with AP[176,177], discrepancies among results finally came to challenge the actual role of PAF antagonism in the clinical setting. Discordance among studies can be partly attributed to the timing of each intervention and/or commercial influence and are further discussed.

Chemokines

So far, only a limited number of experimental and clinical studies have examined the efficacy of chemokine blockade in AP, mostly due to their more distal position within the inflammatory mediator cascade in comparison to cytokines.

In view of IL-8 detrimental effect in experimental AP[178], Osman *et al*[179] investigated the impact of prophylactic blockade of IL-8 in a rabbit model. Interestingly, significant reduction in systemic severity including lung injury, and mortality was observed, whereas the degree of local intrapancreatic damage remained unchanged. Even though, this study could not assess for potential therapeutic effects, the above data strongly supported the role of chemokines in mediating distant organ failure in AP.

Besides IL-8, high concentrations of other chemokines such as MCP-1, growth-related oncogene alpha, and epithelial neutrophil-activating protein 78 could be also found during the early stages of clinical acute pancreatitis. Blockade of specific CXC chemokines *via* specific antibodies, synthetic inhibitors or genetic deletion appeared to reduce pancreatitis associated pulmonary damage in several experimental studies[59,180,181]. Nonetheless, similar to IL-8, a complete absence of effect on local intrapancreatic damage was also observed[59,180,181]. So far, only MCP-1 seemed to exert a detrimental role on the degree of local intrapancreatic damage[182]. Even though, in most of these studies, protective effects of chemokine blockade were observed even in a therapeutic design[59,180,182], no systematic study of their impact upon mortality has ever been carried out.

Adhesion molecules

The expression of adhesion molecules is pivotal for the development of endothelial barrier dysfunction, transmigration of neutrophils and concomitant development of organ dysfunction. Treatment with antibodies against adhesion molecules like ICAM-1 and platelet endothelial cell adhesion molecule-1 (PECAM-1) has shown to be effective in the experimental setting[71,183-185]. Similarly, recent data demonstrate that platelets regulate leukocyte rolling in acute pancreatitis *via* induction of P-selectin, which

was critical in supporting leukocyte rolling in inflamed venules of the pancreas[186]. It seems that inhibition of P-selectin protected against pancreatic tissue injury in experimental pancreatitis[187].

Macrophages

The roles of macrophages in the pathogenesis and progression of experimental AP make these cells interesting therapeutic targets since they exhibit both pro- and anti-inflammatory properties. Macrophages inhibitors (compounds such as gadolinium chloride, liposome-encapsulated dichloromethylene diphosphonate, and PAF antagonists) were shown to modulate the systemic inflammatory response[188]. However, most studies administered the inhibitors before AP was induced, which is clinically less relevant because most patients present after pancreatic injury. Other approaches to modify macrophages, either *in vivo* or *ex vivo*, into cells with anti-inflammatory properties have recently been tested. Currently, favourable effects of IL-4 and IL-13 have only been confirmed *in vitro*[189], while transfer of hemin-activated macrophages promoting production of anti-inflammatory agents seems promising[60,64,190,191]. In a study involving a xenogenic system, human bone marrow-derived clonal mesenchymal stem cells were administered to rats with mild or severe AP[192]. The human bone marrow–derived clonal mesenchymal stem cells induced Foxp3$^+$T regulatory cells and suppressed pancreatic infiltration by T cells. Although more studies are needed in this area of research, stem cell–based immunosuppressive strategies could be developed as allogenic therapies for AP[193].

Corticosteroids, NF-κB and HMBG1

In rat models of AP, hydrocortisone has reduced mortality and blood cytokine levels[194-196]. At the moment, no human trials using steroids as treatment of AP have been published and attempts to show a beneficial effect of steroids as prophylaxis against post-ERCP pancreatitis in prospective placebo-controlled trials have been disappointing[197-199]. The use of glucocorticoids may, however, find a place as part of a combination therapy, as they suppress the inflammatory response, potentially through the inhibition NF-κB[200,201].

Increased levels of NF-κB during acute inflammation correlate well with AP severity[24,25], indicating that potential inhibition could improve outcomes[202,203]. NF-κB signalling seems to regulate autophagy during necrotising pancreatitis, while inhibition of NF-κB pathway reduced serum amylase and autophagosome formation in experimental models[204]. Similarly, lung injury and pancreatic destruction was lower in rats with acute necrotising pancreatitis following administration of NF-κB-*N*-acetylcysteine inhibitor[205].

The complex role of HMG1 in AP has not allowed the conduction of many studies[206]. Previous results have shown that anti-HMGB1 antibody improves lipopolysaccharide (LPS)-induced acute lung injury in mice[51], and ventilator-induced lung injury in rabbits[207]. In AP blockade of HMGB1 has been reported to attenuate the development of severe disease, as well as, associated organ dysfunction[208]. However, even though, HMGB1 can increase the permeability in enterocytic monolayers and bacterial translocation in mice[209], blockade of HMGB1 eventually deteriorated gut barrier function in this study[208].

THE STORY OF IMMUNE-MODULATING THERAPIES:…OR FICTION

Inhibiting pro-inflammatory mediators (*e.g.,* PAF, IL-6, ICAM-1, TLR-4), enhancing anti-inflammatory mechanisms (*e.g.,* IL-10) or modulating cellular immune responses, have all been found to be beneficial in experimental pancreatitis models[71,149,210,211]. Unfortunately, at the moment, they have all failed to find their way from the laboratory bench to the patient's bedside[211,212], with the possible exception of preventing post-ERCP pancreatitis[213,214]. In this respect, a number of issues must be considered and addressed.

First, we have to keep in mind that discouraging results coming from the few representative clinical studies available on single anti-inflammatory agents does not necessarily mean that this approach is flawed in principle. The inflammatory mediators identified so far, most likely represent only the "tip of the iceberg"[215]; a million other mediators, underlying, interacting and regulatory mechanisms awaiting elucidation. Therefore, the concept of blocking single pro-inflammatory mediators could be an over-simplistic strategy to deal with the complex problem of acute pancreatitis, if there is any "ultimate" target at all. Of note, disastrous effects have been noted when single proximal mediators of the inflammatory response were blocked including development of anti-DNA, antinuclear or antithyroid antibodies, leading to various muscoloskeletal, neurological and skin manifestations[216,217].

In the complex network of inflammatory response, a multimodal strategy to inhibit several pro-inflammatory agents instead of one may be more useful[218,219]. The combination of the broad-acting antioxidant *N*-acetylcysteine, monoclonal antibodies against the adhesion molecule PECAM-1 and lexipafant was effective in animals with organ failure associated with AP[220]. The acute phase response and organ dysfunction was decreased, while gut barrier failure and translocation was prevented[220]. However, even in the case of multimodal strategy the example of TNF and anti IL-1 that share similar characteristics in both their pathophysiological functions, as well as, their regulation is worth noting[134,221]. As it was convincingly shown by Denham *et al*[131], no additive protective effects could be demonstrated by combined genetic disruption the IL-1 receptor and TNF in a murine model of AP, reflecting the huge challenges lying beyond the functional redundancy of the immune system[222].

However, even in such cases of multimodal management the timing of intervention remains critical. It is evident that the window for anti-inflammatory therapy

to suppress excessive immune activation is very limited. Experimental and clinical evidence shows that the time limit for efficacious medical treatment is no more than 60 h from the onset of the symptoms of AP[223]. In fact, as stated by at least two controlled clinical trials including the European PAF-antagonist phase Ⅲ trial[175,224], beneficial effect was achieved when treatment was introduced no later than 48 h after the onset of symptoms. Even though further trials are pivotal to clarify the proper timing for intervention, efforts are hampered by the fact that many patients present at a late stage of the disease, when organ failure may already be present and the patient may be already on his way to CARS or even immunosuppression[175].

This data challenges us to further explore and understand the tight balance and mechanisms underlying SIRS, CARS or even a mixed inflammatory response syndrome during the time-course of acute pancreatitis. In the last few years, an effort to monitor defects in monocyte function, as those reflected in reduced expression of HLA-DR during severe AP is under progress[107,225,226]. However, inflammatory stages may not be synchronous in the same patient, and even though immune-suppression may be evident in the peripheral blood, other end organs including the lungs may still be in the pro-inflammatory stage. Therefore, immune-stimulatory treatment must be used with caution and physicians should have the proper means to monitor the patients' immune-inflammatory state, in order to most accurately identify patients who are at risk of organ failure. Signalling pathways and molecules of circulating leukocytes including HLA-DR, NF-κB, signal transducers and activators of transcription, and members of mitogen activated protein kinase family represent good candidates that could serve as future indices to identify the patients at risk for secondary infections and, thus, late organ failure[227-229].

Nonetheless, currently progress in AP research is slow, mainly due to the inaccessibility of the human pancreas to direct observation or biopsy, as well as, the self-destructive nature of the disease itself which does not allow distinguishing initiating events from the concomitant or consequent inflammatory response. Flaws in study design, including small sample sizes, variable tools to stratify disease severity and non-comparable study endpoints further hinder understanding of this complex disease. Consequently, most of our knowledge comes either from circulating inflammatory mediators and cells or animal models that are inevitably unable to simulate the complexity and individuality of human condition[230-232]. As a result, the authors of this review note a relative gap in experimental but mostly clinical research pertaining to AP during the last decade. The latter is reflected in the year of publication of many of our references, in contrast to the flourishing field of animal model pancreatitis during the 90's and the dawn of the new millennium.

The key to future advances lies in obtaining data upon actual patients, making use of correct scientific methods and better design of clinical trials[233]. Recording of other meaningful parameters besides mortality including time from the onset of symptoms to type of intervention, permanent target organ damage, quality of life, pain scores or hospital stay should also be recorded. Improvement of the communication of the results is also pivotal. Scientific and editorial community must share the responsibility of publishing well-designed and well-conducted clinical studies irrespective of commercial or financial influence. Examples of poor management of these issues could be partly mirrored by the controversial efficacy of protease inhibitors in human AP[234] as well as, the highly debated results following lexipafant administration[235].

CONCLUSION

Treatment of AP by immune modulation currently represents an attractive and highly promising concept. However, further meticulous work lies ahead in order to overcome the fundamental conceptual problems surrounding the complex pathophysiology of this challenging disease. Individualized and timely management calls for close monitoring so that best possible outcomes are ensured for our patients.

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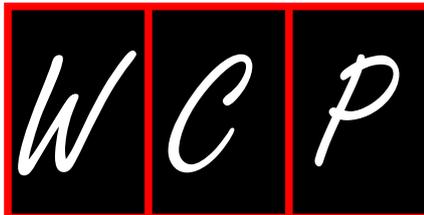
Enteral nutrition and immune modulation of acute pancreatitis

Refaat A Hegazi, Tiffany DeWitt

Refaat A Hegazi, Tiffany DeWitt, Research and development division, Abbott Nutrition, Columbus, OH 43219, United States
Author contributions: Hegazi RA and DeWitt T have been equally involved in reviewing the literature and writing the manuscript.
Correspondence to: Refaat A Hegazi, MD, PhD, MPH, MS, Medical Director Research and Development, Abbott Nutrition, 3300 Stelzer Rd, Columbus, OH 43219,
United States. refaat.hegazi@abbott.com
Telephone: +1-614-6247811 Fax: +1-614-7277811
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Abstract

Enteral nutrition has been strongly recommended by major scientific societies for the nutritional management of patients with acute pancreatitis. Providing severe acute pancreatitis patients with enteral nutrition within the first 24-48 h of hospital admission can help improve outcomes compared to parenteral nutrition and no feeding. New research is focusing in on when and what to feed to best improve outcomes for acute pancreatitis patients. Early enteral nutrition have the potential to modulate the immune responses. Despite this consistent evidence of early enteral nutrition in patients with acute pancreatitis, clinical practice continues to vary due to individual clinician preference. Achieving the immune modulating effects of enteral nutrition heavily depend on proper placement of the feeding tube and managing any tube feeding associated complications. The current article reviews the immune modulating effects of enteral nutrition and pro- and prebiotics and suggests some practical tools that help improve the patient adherence and tolerance to the tube feeding. Proper selection of the type of the tube, close monitoring of the tube for its placement, patency and securing its proper placement and routine checking the gastric residual volume could all help improve the outcome. Using peptide-based and high medium chain triglycerides feeding formulas help improving feeding tolerance.

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Core tip: Due to the decreased food intake and increased nutrient requirements, patients with acute pancreatitis are at increased risk of malnutrition. Beyond meeting calorie and protein requirements, enteral nutrition exerts an immune modulating effect on the intestinal and systemic immune responses. Achieving the beneficial effects of enteral nutrition requires proper selection, placement and management of the feeding tubes and proper selection of the feeding formula. This review highlights new research of the immune effects of enteral nutrition, probiotics and prebiotics and suggests tools to help improve the patient adherence and tolerance to tube feeding.

INTRODUCTION

The majority of acute pancreatitis (AP) episodes are considered mild or moderate. However, up to a third of patients with AP present with either severe acute pancreatitis (SAP) (defined as either infected (peri)pancreatic necrosis or persistent organ failure) or critical AP (defined as both infected (peri)pancreatic necrosis and persistent organ failure) according to a newly published consensus

Table 1 Current nutrition practice guidelines of enteral nutrition in patients with severe acute pancreatitis	
Association	**Recommendation for nutritional care of SAP (Grade)**
International Consensus Guideline Committee[28]	EN is generally preferred over PN, or at least EN should, if feasible, be initiated first. (Grade A: Platinum)
	For EN, consider small peptide-based, medium chain triglyceride oil formula to improve tolerance. (Grade B: Gold)
European Society of Parenteral and Enteral Nutrition[29]	In severe necrotizing pancreatitis, EN is indicated if possible (A)
	Peptide-based formula can be used safely in AP (A)
	Standard formula can be tried if they are tolerated (C)
ASPEN/SCCM 2009 Critical Care Guidelines[30]	Patients with severe acute pancreatitis may be fed enterally by the gastric or jejunal route. (Grade: C)
	Tolerance to EN in patients with severe acute pancreatitis may be enhanced by the following measures:
	Changing the content of the EN delivered from intact protein to small peptides, and long-chain fatty acids to medium-chain triglycerides of a nearly fat-free elemental formulation. (Grade: E)
American College of Gastroenterology[31]	In severe AP, EN is recommended to prevent infectious complications
	Parenteral nutrition should be avoided unless the enteral route is not available, not tolerated or not meeting caloric requirements (strong recommendation, high quality of evidence)

SAP: Severe acute pancreatitis; EN: Enteral nutrition.

classification of the severity of AP[1]. SAP is a common cause of systemic inflammatory response syndrome (SIRS), a serious complication that is associated with multi-organ failure, increased risk of infections and mortality and mediated by increased expression of pro-inflammatory cytokines and chemokines[2]. In addition to this inflammatory and catabolic stress, the gastrointestinal symptoms patients with AP present with (abdominal pain, vomiting and diarrhea) pose an even more increased risk of malnutrition. Enteral nutrition (EN) exerts immune modulating effects in patients with AP beyond meeting the caloric and protein requirements. The current article reviews the nutritional issues of patients with AP and explores the potential immune modulating role of EN and nutrients.

IMMUNE MODULATING EFFECTS OF ENTERAL NUTRITION

Compared to parenteral nutrition (PN), the use of enteral nutrition in patients with SAP has been shown to improve clinical outcomes, decrease infective complications and reduce the incidence of multiple organ failure in patients with SIRS[3]. The exact mechanism of these beneficial effects of enteral nutrition in patients with SIRS remains to be determined. Previous studies to explain these effects suggest immunomodulatory effects of enteral nutrition on both the systemic and intestinal mucosal immune systems. The integrity of the intestinal epithelial and immune cells of the gut-associated lymphoid tissue and the intestinal barrier plays an important role in maintaining intestinal homeostasis and preventing bacterial translocation[4]. The intestinal epithelial cells (IEC)-derived cytokine secretion plays a major role not only in maintaining intestinal mucosal functions but also in the maturation and optimum functions of lymphocytes. Enteral nutrients play a major role in maintaining the integrity of IEC. For instance, duodenal infusion of the amino acid glutamine induced the expression of the major cytoprotective enzyme, heme-oxygenase-1 (HO-1).

HO-1 is an important enzyme for immune homeostasis and exerts anti-inflammatory effects in animal models of intestinal inflammation[5].

The literature has consistently shown that EN is preferred to PN in patients with SAP and therefore EN was recommended by major gastroenterology, critical care and nutrition societies (Table 1). Consistently, Wu *et al*[6] (2010) conducted a randomized trial to determine the effects of EN compared to PN in preventing pancreatic necrotic infection in patients with SAP. EN patients experienced significantly less ($P < 0.05$) necrosis, surgery related complications and mortality compared to the PN group[6]. The most common cause of mortality (27%) was multiple organ failure from sepsis impacting 43% of patients on PN and 11% on EN ($P < 0.05$)[6].

The beneficial immune, hormonal and endocrine effects of EN on the intestinal mucosa make it superior to long-term starving patients with mild and moderate acute pancreatitis. Consistently, a recent randomized controlled trial showed that patients receiving EN within 24 h of hospital admission had significantly reduced intensity and duration of abdominal pain, need for opiates, and risk of oral food intolerance as compared to the no-feeding group, with no difference in hospital length of stay[7].

For EN to exert its immune and other beneficial effects, the patient's tolerance to the fed formula is key. Tube feeding associated intolerance is common, occurring in approximately 50% of tube-fed patients. Due to the associated exocrine pancreatic insufficiency, patients with SAP are at even higher risk of feeding intolerance. The nutrient composition of EN formulas may help enhance the tolerance to the formula and increase the likelihood of adherence for patients to achieve their goal feeding. Consistently, major clinical and scientific societies recommend feeding patients with SAP with peptide based and high medium chain triglycerides formulas (Table 1). Interestingly, medium chain triglycerides have been shown to exert anti-inflammatory effects in animal models of inflammatory bowel diseases[8].

EARLY *VS* DELAYED ENTERAL NUTRITION

We previously reported that early initiation of jejunal feeding (within 24 h of consulting) and reaching early goal tube feeding were associated with less duration of stay in the intensive care unit independent of the APACHE Ⅱ scores[9]. Consistently, a retrospective analysis of predicted SAP patients early EN (< 48 h) was superior to delayed EN (> 48 h) in the prevention of infected necrosis and mortality[10]. Akin to SAP, early EN is preferred to late EN in critically ill and surgical patients[11]. Recently, Sun *et al*[12] investigated the impact of early EN on the immune function and clinical outcomes. The single-center, prospective, randomized controlled trial analyzed 60 patients with SAP. One group (*n* = 30) received EN within 48 h of admission and the second group received TPN days 1-7 and then started EN on day 8. At day 7, difference were seen in the immune parameters between the two groups with the early EN group having significant differences in CD4+ T-lymphocyte percentage, CRP levels, HLS-DR expression and IgG levels (*P* < 0.05). No significant differences were seen in CD4+/CD8+, CD8+ T-lymphocyte percentage, IgM or IgA. The authors suggest that early EN in SAP patients may play a role in moderating the excessive immune response that is seen in the early stages of SAP. Significant decreases in ICU stay, pancreatic infections, MODS and SIRS were seen in the early EN group. There was no difference seen in hospital mortality or surgical operations between the two groups. While this study reported on early EN compared to delayed EN, the data reported is comparing early EN to exclusive TPN in days 1-7 of hospital stay.

While research continues to support early EN in SAP patients, there is an ongoing discussion of the optimal tube type selection that allows patients to reach goal feeding rates while minimizing stimulating the exocrine pancreatic secretions. In 2012, Singh *et al*[13] conducted a randomized, parallel-group, active controlled trial to determine if there was a difference in clinical outcomes for patients fed nasogastric (NG) *vs* nasojejunal (NJ). A pilot study had previously suggested that there were no differences in clinical outcomes[14] and this larger study further supports those findings[13]. There was no significant difference seen between NG and NJ groups in pain in mortality, refeeding, length of hospital stay or intestinal permeability[13]. The NG group did experience significantly higher rates of any one infectious complication compared to the NJ group (95%CI)[13]. Tube placement either NG or NJ in AP patients can positively impact the patient. We have previously shown that a double-lumen nasogastric decompression and jejunal feeding tube system (NGJ) is a safe conservative management for patients with gastric outlet obstruction reducing the need for surgery and PN[15].

Interestingly, despite the prevailing evidence of the clinical outcome benefits of EN in patients with AP, physician preference for PN is still a reality leading to many unnecessary PN orders. A study in Australia and New Zealand by Davies *et al*[16] in 2011 determined that the most common reasons patients received PN were preference of the treating intensivist (38%) or surgeon (22%). In this prospective observational multicenter study, 42% of the patients received PN and that PN was more frequently the initial therapy compared to EN[16]. Some myths and fears of initiating tube feeding in patients with SAP may have contributed to these observations. Having the technical capabilities of not only placing the enteral tubes but more importantly managing them is key to implementing a successful tube feeding strategy at any certain setting. For instance, tube displacement, a complication that could lead to the risk of aspiration, should be managed by radiographic confirmation of the position of the tip of tube and routine follow up by a dedicated nutrition therapy team. We have previously shown that devices like nasal bridles could help maintaining the tubes in place[17]. Certain types of tubes like the NGJ tube system is another tool that can help address the problems of monitoring and managing the gastric residual volume while maintaining enteral feeding.

IMMUNE MODULATING EFFECTS OF PREBIOTICS AND PROBIOTICS

The intestinal luminal micro biota plays an important role in the pathogenesis of SAP-associated infections. It was hypothesized that the gut is the "undrained abscess" in patients with SAP[18]. Microbial analysis of peri-pancreatic fluid collections reveal that the source of these microbial translocation is likely the intestinal lumen[18]. Therefore, modulating the milieu of the intestinal microbes into the more beneficial strains had been the target of years of research. Probiotics are the exogenous microbes that when given orally exerts some benefits to the host. Prebiotics are non-digestible dietary carbohydrates fermented by the intestinal microbes the byproducts of which stimulate the proliferation of the beneficial intestinal microbes or enhance their metabolic activities. Pre and probiotics have been hypothesized to possibly play a role in AP by modulating the gut micro biota to decrease bacterial translocation and reduce the associated infections. Prebiotics have been previously reported to be beneficial to the care of SAP patients by normalizing APACHE Ⅱ and CRP levels[19]. However, the study by Besselink *et al*[20] (2008) casted some doubt on the beneficial role of probiotics. To summarize, Besselink *et al*[20] randomized 298 patients with predicted SAP into two groups. The intervention group received a probiotics mix of probiotics strains and was compared to a placebo group. Both groups received enteral feeding. The primary endpoint of the study, infectious complications, was not significantly different between the two groups and mortality was higher in the probiotics group. However, this study has some limitations that were previously discussed in detail[21,22]. For instance, questions were raised regarding the lack of clinical studies demonstrating the safety of the specific probiotics mix and doses used in the study. At baseline, gut ischemia was

more common in the probiotics group raising a concern of selection bias. Moreover, both study groups received a prebiotics-supplemented enteral formula. It could be questioned whether the bifidogenic effects of these prebiotics had resulted in "iatrogenic bacterial overgrowth" in the probiotics group or that the outcome of the study could have been affected by a more favorable effect in the control group. Sharma *et al*[23] looked at the role of probiotics on gut permeability and endotoxemia to prevent infectious complications in AP patients. The investigators enrolled 50 patients into a double-blind, randomized placebo controlled trial. Due to results of the aforementioned study by Sharma *et al*[23] was abandoned the study after only enrolling 50 patients. The analysis of the 50 patients did not show any effects of probiotics in helping maintain gut integrity to prevent infectious complications. The authors suggest that probiotic use is inappropriate in the routine management of AP.

Research continues in the area of probiotics in SAP patients to help better understand which strains of probiotics may prove to possess clinical benefits. One interesting concept is to combine probiotics with EN. In 2013, Wang *et al*[24] studied the effects of ecoimmunonutrition (adding combined live *Bacillus subtilis* and *Enterococcus faecium* enteric-coated probiotics to EN) on gastric motility and cytokine production in patients with SAP. The study included 183 patients who were randomized to receive TPN, EN or ecoimmunonutrition. Compared to TPN, EN and ecoimmunonutrition significantly decreased plasma TNF-α and IL-6 levels ($P < 0.05$) with the ecoimmunonutrition group seeing even further decreases at days 7 and 14 compared to the EN group ($P < 0.05$). At days 7 and 14, the anti-inflammatory cytokine IL-10 levels were significantly increased in both EN and ecoimmunonutrition groups compared to the TPN group ($P < 0.05$) with the ecoimmunonutrition group again showing greater increases compared to the EN group ($P < 0.05$). The study was also able to detect significant differences in the occurrence of pancreatic sepsis, MODS and mortality among the three groups. The EN and the ecoimmunonutrition group had significantly less rate of sepsis, MODS and mortality compared to TPN ($P < 0.005$) and the ecoimmunonutrition group had significantly less levels of markers of inflammation compared to the EN group.

Akin to the ecoimmunonutrition is the synbiotics approach of combining probiotics and prebiotics. Studies of the effects of synbiotics in the management of patients with acute pancreatitis have been previously investigated and showed promising results. Olah and colleagues randomized 45 patients with acute pancreatitis into two groups[25]. Both groups received jejunal feeding of isocaloric feeding formulas supplemented with 10 grams of oat fiber. The treatment group (22 patients) received live lactobacillus plantarum 299, and the control group (23 patients) received a similar dose of heat-inactivated lactobacillus plantarum 299. The rate of pancreatic infection was significantly lower in the synbiotics group than the control group (30% *vs* 5 %, $P < 0.05$). Moreover, patients required significantly less surgical interventions in the synbiotics group as compared to the control group (22% *vs* 5%, $P < 0.05$)[25]. The same investigators also studied the effectiveness of another group of synbiotics (a mix of 4 different lactobacillus genera and 4 plant fibers)-supplemented jejunal feeding in patients with severe acute pancreatitis[26,27]. Consistently, the study showed that septic complications (infected pancreatic necrosis or abscesses) were significantly lower in the synbiotics group. The inherent composition of individual patient's intestinal micro biota could play a role in determining the effects of the different strains of probiotics and type of prebiotics. Identifying these profiles of intestinal micro biota could help selecting the right combination of probiotics and/or prebiotics for the right patient, an interesting area for future research.

CONCLUSION

Patients with acute pancreatitis are at increased risk of malnutrition due to both decreased food intake and increased requirements as a result of the associated inflammatory disease. In addition to meeting calorie and protein requirements, enteral nutrition exerts an immune modulating effect on the intestinal and systemic immune responses. Enteral nutrients, prebiotics and probiotics are important for the optimal function of the intestinal epithelial cells and maintaining the intestinal micro biota homeostasis. Achieving the beneficial effects of enteral nutrition requires proper selection, placement and management of the feeding tubes and proper selection of the feeding formula.

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Surgical management of necrotizing pancreatitis: An overview

George Kokosis, Alexander Perez, Theodore N Pappas

George Kokosis, Alexander Perez, Theodore N Pappas, Department of Surgery, Duke University Medical Center, Durham, NC 27705, United States

Correspondence to: Theodore N Pappas, MD, Department of Surgery, Duke University Medical Center, Durham, NC 27705, United States. pappa001@mc.duke.edu
Telephone: +1-919-6813442 Fax: +1-919-6681826

Abstract

Necrotizing pancreatitis is an uncommon yet serious complication of acute pancreatitis with mortality rates reported up to 15% that reach 30% in case of infection. Traditionally open surgical debridement was the only tool in our disposal to manage this serious clinical entity. This approach is however associated with poor outcomes. Management has now shifted away from open surgical debridement to a more conservative management and minimally invasive approaches. Contemporary approach to patients with necrotizing pancreatitis and/or infectious pancreatitis is summarized in the 3Ds: Delay, Drain and Debride. Patients can be managed in the intensive care unit and any intervention should be delayed. Percutaneous drainage can be utilized first and early in the course of the disease, followed by endoscopic drainage or video assisted retroperitoneoscopic drainage if necrosectomy is deemed necessary. Open surgery is now less frequently performed and should be reserved for cases refractory to any other approach. The management of necrotizing pancreatitis therefore requires a multidisciplinary dynamic model of approach rather than being a surgical disease.

Core tip: This is a review of the most current literature in management of necrotizing pancreatitis and infected necrotizing pancreatitis. The recent years more conservative management has been advocated. Additionally, if necrosectomy is required, minimally invasive approaches such as endoscopic, laparoscopic, or video assisted retroperitoneoscopic debridement are gaining popularity over the traditional open surgery. This paper illustrates this paradigm shift and can help guide the multidisciplinary teams when treating patients with severe acute pancreatitis.

INTRODUCTION

Acute pancreatitis currently accounts for more than 200000 hospital admissions every year in the United States[1,2]. In most cases, acute pancreatitis represents a mild, self-limited disease but in 15%-25% severe acute pancreatitis (SAP) develops, manifested with pancreatic parenchymal and/or peri-pancreatic tissue necrosis[3]. Pancreatic necrosis accounts for substantial additional morbidity, with mortality rates remaining as high as 10%-20% despite advances in

critical care[4,5]. The clinical course of SAP is divided in two phases. An early inflammatory phase that lasts the first 2 wk and a late phase after the first 2 wk, marked by infectious complications. Mortality rates in the event of infected necrotizing pancreatitis increase up to 30% with surgical intervention and nearly 100% in the absence of any intervention[6,7].

Historically the only tool in our disposal was laparotomy therefore early open surgical intervention for extensive pancreatic necrosis had been broadly adopted. This previously held dogma has now matured into a dynamic and multi-modal management strategy. The volume of open surgical debridement has dramatically fallen over the years as the minimally invasive techniques prove to be effective. This paper will review the current trends in intervention for the treatment of necrotizing pancreatitis and infected pancreatic necrosis.

PATHOLOPHYSIOLOGY, CLASSIFICATION, AND CLINICAL COURSE OF NECROTIZING PANCREATITIS

Acute pancreatitis is most commonly caused by gallstones or alcohol, with less common etiologies including tumor, trauma, hypertriglyceridemia, medications (*e.g.*, azathioprine, furosemide, steroids, cimetidine) and iatrogenic injuries (*e.g.*, endoscopic retrograde cholangiopancreatography and surgery)[8]. The pathogenesis is initially caused by unregulated activation of trypsin within the pancreatic acinar cells. After activation of trypsinogen to trypsin, several enzymes such as elastase, phospholipase A2 and the complement and kinin are activated. The release of these enzymes and the resulting injury to the pancreatic parenchyma triggers an inflammatory cascade resulting in additional cytokine production, including interleukin (IL)-1, -6 and -8, as well as tumor necrosis factor α[1]. Additionally, activation of endothelial cells enables the migration of leukocytes with release of more injury inducing enzymes. The endpoint of this cascade is a systemic inflammatory response syndrome (SIRS), characterized by loss of vascular tone, systemic vascular resistance and increased capillary permeability with third spacing of plasma volume, leading to hypotension. SIRS can produce adult respiratory distress syndrome and multiorgan dysfunction syndrome.

The Atlanta Symposium held in 1992 was a landmark consensus that established a clinically based classification system for acute pancreatitis[9]. The advancement in diagnostic imaging and understanding of pathophysiology through ongoing basic science research led to revisions throughout these years. The sepsis related organ failure assessment (SOFA score), was an alternative soring system increasingly applied for predicting outcome based on the degree of multiorgan failure[10]. More recently, multidisciplinary consensus panels have recommended revisions to further globalize the definitions of acute

pancreatitis and the clinical entities associated with it[3,11]. Of all the above entities, necrotizing pancreatitis most commonly manifests as necrosis involving both the pancreatic and peripancreatic tissues and less commonly the pancreatic or peripancreatic tissues alone[3]. Acute necrotic collections occurring in necrotizing pancreatitis are heterogeneous collections with varying amounts of fluid, usually occurring less than 4 wk after the onset of acute pancreatitis. Walled off necrosis occurring in the context of necrotizing pancreatitis, has the same above characteristics with acute necrotic collections but occurs 4 or more weeks after the onset. Most of the evidence suggests no absolute correlation between the extent of necrosis and the risk for infection or duration of symptoms, although this is still controversial[11,12]. Contrast enhanced computed tomography (CECT) provides the highest accuracy for necrotizing pancreatitis when performed after the first week[1,13]. Fine needle aspiration under radiologic guidance has been widely used in the past, however its clinical relevance has diminished and its utilization is no longer recommended as a necessary diagnostic tool[14].

The natural history of necrotizing pancreatitis is variable as it may remain solid or liquefy, remain sterile or become infected over time[11]. The first 2-4 d after the onset of acute pancreatitis are the most important when about 15%-25% of patients takes the course of a severe disease. If necrosis occurs, it is usually characterized by two phases. During the first phase, occurring the first 2 wk, a systemic inflammatory response is predominant, which is often associated with multiple organ failure, especially after the first 72 h, conferring to 50% of the mortality. In the second, late phase that starts 14 d after the onset of symptoms, the systemic inflammation often regresses and infected necrosis occurs in about 30% of patients with necrotizing pancreatitis[15,16]. The bacteriological analysis of the fluid reveals predominantly gut flora, as *Escherichia coli*, *Enterococcus*, *Klebsiella*, however, *Staphylococcus aureus*, and candida species have been observed[12,17].

SURGICAL MANAGEMENT OF NECROTIZING PANCREATITIS AND COMPLICATIONS

The early management of severe acute pancreatitis and necrosis is of great importance and should take place in the intensive care unit, mainly consisting of vigorous resuscitation to overcome the substantial third spacing resulting from peripancreatic inflammation and capillary leak. Administration of antibiotics in case of pancreatic necrosis without documented infection remains a controversial area. Prophylactic antibiotics were generally recommended in the past but more recently, randomized studies have failed to show clear benefit. Although current literature does not support use of prophylaxis in all cases of severe acute pancreatitis, early empiric use in patients with clinical signs of infection (fever, leukocytosis, hemodynamic instability) is clearly advocated[18,19].

Indication for surgical intervention is when there is documented or suspected infection. The presence of infection can be established with a positive computed tomography (CT) guided FNA although it is not the standard of care. Infection can be presumed with the presence of extraluminal gas in the pancreatic or peripancreatic tissues on CECT. Patients without documented infection and with clinical deterioration, SIRS, and MOFS are no longer thought to be immediate candidates for surgical intervention and surgery is reserved as the last resort[3,16,20]. In particular, the first week of acute pancreatitis characterized by SIRS has very poor prognosis regardless surgical intervention[16]. Emergent surgery regardless the timing is indicated in case of abdominal compartment syndrome and intestinal perforation as a result of fulminant necrotic pancreatitis[21]. Sterile acute necrotic collection will require surgical intervention only in the presence of significant mechanical obstruction, such as biliary and gastric outlet obstruction and failure to thrive[3].

Available methods for intervention include the open approach, the minimally invasive approaches with percutaneous catheter placement, laparoscopic and retroperitoneoscopic approach, endoscopic and lastly hybrid approaches that will be analyzed below.

Open surgical approach

Although open surgery during the early phase can be associated with mortality rates up to 65%, randomized data confirms the benefit of late surgical intervention (at least 12 d after the onset of symptoms) with decrease of mortality to 27% and even lower between 10%-20% in specialized centers[22,23]. Necrosectomy is performed either through a subcostal or a midline longitudinal incision. The retroperitoneum is entered through the lesser sac and the pancreas is exposed. In cases that the above approach is not feasible, infracolic approach has been described as alternative. Debridement is typically performed with blunt finger dissection or ring forceps representing an organ-sparing technique[3,22]. Formal resection is avoided to minimize the incidence of bleeding, fistulae and removal of vital tissue[3]. Enterotomies are avoided, again to decrease the incidence of post-operative enterocutaneous fistula[4]. Cholecystectomy can be added to the procedure in cases of gallstone pancreatitis[24]. The area of necrosectomy is irrigated with several litters of saline.

Two distinct open surgical completion techniques have been described: (1) open abdominal packing, with return trips to the operating room every 48 h for further debridement until granulation tissue has replaced the retroperitoneal necrosis, a processes called "marsupialization". Some authors have described the "sandwich technique" were suction tubes were placed for superficial drainage and the wound was covered by protective materials (Opsite dressings) and a mesh was interposed between the edges of the fascia[25,26]. All reoperations can be made in the surgical intensive care unit (ICU). Wounds were permitted to heal by secondary intention; and (2) continuous post-operative lavage. This technique involves insertion of two

or more double lumen Salem® sump tubes (20-24 French) and single lumen silicone rubber tubes (28-32 French) through separate incisions with their tips in the lesser sac and necrotic areas. The smaller lumen tubes are used as the inflow and the larger lumen tubes for outflow. Thirty five to forty litters of fluid are used for lavage. Drains can be removed within 2-3 wk[27,28]. Alternatively, "closed packing" is similar to continuous lavage, but also involves multiple, large gauze-filled Penrose® drains that pack the abscess cavity and control minor bleedings. Drains can be removed after a minimum of 7 d[6,28,29].

The above techniques are associated with complications in the immediate post-operative period as well as long term. Potential immediate complications include hollow viscus perforation, organ failure, infection, wound dehiscence and end organ failure such as renal failure. Bleeding is rare and can be managed angiographically[5]. Long-term complications include incisional hernias, gastrointestinal fistula, gastric outlet stenosis, colonic and pancreatic fistulas. All the above are more common with the open techniques. Additionally, exocrine and endocrine pancreatic insufficiency is another known long term complication. Morbidity varies between studies and rates 34%-95% have been reported[3]. Mortality averages between 10%-20% in most studies[6]. Between the two above-mentioned open techniques, the closed continuous lavage is most commonly used[30].

Percutaneous therapy

In 1998, Freeny *et al*[31] first described image guided percutaneous catheter drainage (PCD) to temporize sepsis and half of the patients included in the study were treated with the above technique as the only intervention. Since then, PCD has progressively become more popular as a first line treatment. The minimally invasive nature of this technique allows intervention even in the early phase of severe necrosis, when an open approach would be associated with increased mortality. It can be used as the primary treatment, as an adjunct to other techniques, or to reduce post-operative persistent fluid collections[3]. With preferred retroperitoneal approach through the left flank, catheters of size 12-30 French are placed with the guidance of CT or ultrasound. Saline flushes are used every 8 h[32,33]. The largest study to date, to review the percutaneous technique comes from van Baal *et al*[34] in 2011. Eleven studies, including 384 patients were analyzed and revealed infected necrosis in 70.6% of the patients treated with PCD and organ failure in 67.2%. No additional surgical necrosectomy was required in 55.7%. Indications for PCD in the above studies were culture proven infected necrosis or clinical deterioration despite maximal medical management. PCD as the first step in a step-up approach was studied in a randomized control trial that will be discussed further in this article. In 33% of the patients included in this study PCD was the only approach[35]. Mortality associated with this technique is found to be about 20%[32]. Morbidity averages at 28% with most common complications being colonic perfora-

tion, intra-abdominal bleeding, gastrointestinal and internal and external pancreatic fistula[34].

Laparoscopic approach

Laparoscopic approach for pancreatic necrosectomy is not so widely advocated and no large series or randomized studies are available. Parekh in 2006 described a laparoscopic technique utilizing 3 ports and a hand port for infra-colic approach and blunt dissection with the fingers or with an endo-dissector and several drains left in 19 patients. Indications were mainly, documented necrosis, progressive organ failure, or persistent symptoms. Success rate was 77% but mortality was 11% with morbidity rate reported 58% mainly including pancreatic fistula, central line infections and clostridium difficile infection. Advantages of this minimally invasive technique are less wound infections and risks include dissemination of retroperitoneal infection into the retroperitoneum. Specialized centers have reported laparoscopic drainage of necrotic collections, once they are walled off, either in the stomach or the small bowel, but this technique is not widely used, due to the technical challenge associated[36].

Retroperitoneoscopic approach

This approach is a modified laparoscopic approach and includes a constellation of modified techniques that utilize a percutaneous tract, usually created under CT guided drainage[37]. This tract is dilated so that a rigid nephroscope, endoscope or even a laparoscope is advanced to provide direct visualization of the necrosis. Then an incision is made through a left translumbar approach[37-39] or a small subcostal incision (5-7 cm)[40] and debridement and lavage is performed until resolution of the necrosis. The term widely used to describe all the above is video assisted retroperitoneal debridement (VARD) and previously used terms as sinus tract endoscopy. Horvath *et al*[41], in 2010, performed a multicenter prospective study to evaluate the safety and efficacy of VARD utilized in 40 patients with infected pancreatic necrosis diagnosed by FNA. A retroperitoneal percutaneous drain was placed within 48 h of admission and was upsized every 3-4 d until a 20 French drain was reached and that was eventually used as the VARD route. From the 40 patients initially enrolled, 25 underwent VARD and 81% required only one trip to the OR (success rate). Patients crossing over to open surgery were found to have a central collection with inferior extension to the mesenteric root, therefore not amenable to drainage or VARD through the required retroperitoneal approach. The authors reported the associated morbidity, including 6% hemorrhage, 10% enteric fistulas and no mortality. Overall in the literature an average success rate is reported as high as 88%, mortality ranges from 0%-20% and peri-procedural morbidity 10%-30%[3,32,33,38,39].

Endoscopic approach

Endoscopic necrosectomy is widely used for infected pancreatic necrosis as a means of a minimally invasive approach. It utilizes moderate sedation (midazolam or propofol and fentanyl) and endoscopy to advance an endoscope in the stomach. Approach to the area of necrosis can be performed either through the stomach or the duodenum. Puncture of the fluid collection can be made either directly by visualizing a bulge or with endoscopic ultrasound (EUS) guidance with the latter being more technically successful and with less adverse effects[42,43]. The collection is punctured with a 19-gauze needle and a guide-wire is advanced under fluoroscopic guidance. The tract is balloon dilated up to 8mm. Then, 2 or more double pigtail plastic stents are placed and the collection is irrigated with 1 liter of normal saline per 24 h. Necrotic tissue is evacuated with a basket, a net or a polypectomy snare[44]. A systematic review of endoscopic necrosectomy of pancreatic necrosis by Haghshenasskashani *et al*[45] in 2011 revealed an overall 76% definitive resolution with endoscopic techniques alone, with a median of 4 sessions. Mortality was 5% and morbidity about 30%, with most common bleeding. Fatal air embolism has been reported in a multicenter study of transluminal endoscopic necrosectomy in Germany (the GEPARD study) and therefore carbon dioxide is now more commonly used for insufflation rather than air[46]. Additionally, this study reports success rate of 80% after a mean of 6 sessions with mortality 7.5% and morbidity 26%. Bakker *et al*[44] in 2012 published a randomized trial comparing endoscopic transgastric necrosectomy *vs* surgical necrosectomy for infected necrotizing pancreatitis. Twenty-two patients were randomized in the study, twelve in the surgical arm and ten in the endoscopic. The endoscopic necrosectomy reduced the post-procedural inflammation as measured by the IL-6 levels, especially the first 24 h. This was also reflected in the significantly lower new-onset multi-organ failure (0% *vs* 50%) and pancreatic fistulas (10% *vs* 70%). Additionally, mortality and major morbidity was reduced in the endoscopic group when compared to the open (20% *vs* 80%).

Hybrid approach

More recently, "step-up" approaches in managing infected pancreatic necrosis are gaining more popularity. This approach utilizes a percutaneous drain or endoscopy to mitigate sepsis. If drainage fails to control sepsis, the next step is minimally invasive retroperitoneal necrosectomy, VARD or sinus tract endoscopy. The rationale behind this approach is the aim to control the sepsis, rather than complete removal of the infected necrosis. This reduces the rate of complications and death my minimizing the surgical trauma and the inflammatory response to a surgical intervention in already critically ill patients. This approach also addresses the challenge of intervening early in the course of necrotizing pancreatitis (first week) that is associated with increased mortality. Percutaneous drains to control sepsis are used, instead of open necrosectomy. The PANTER study, published by van Santvoort *et al*[35] and Besselink *et al*[47] in 2010, established a paradigm shift in managing infected necrotic pancreatitis with a
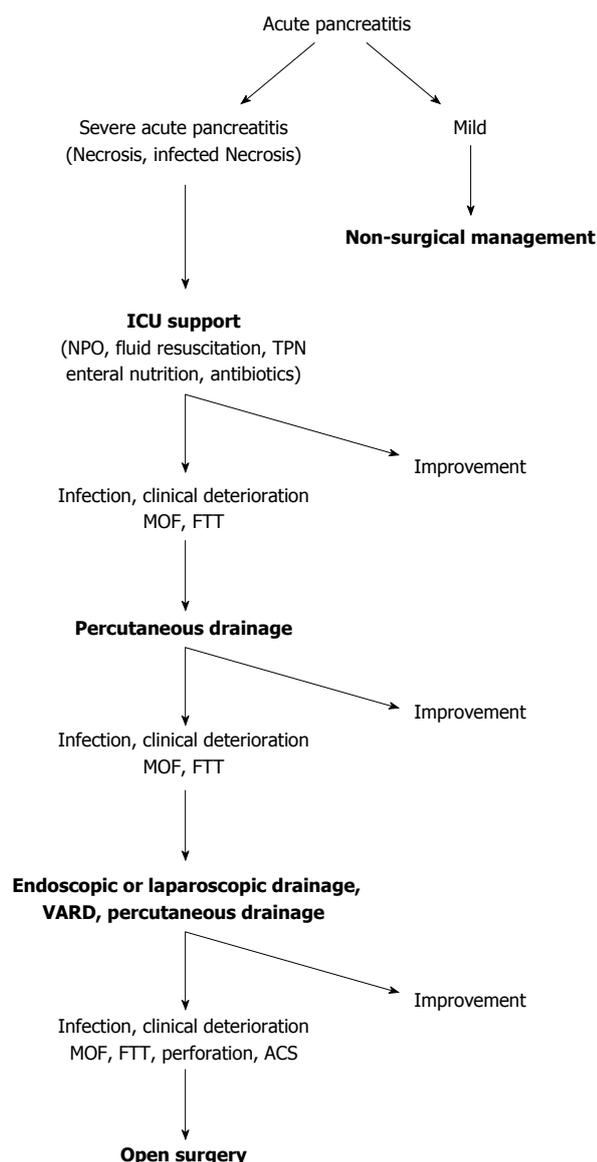
Figure 1 Treatment algorithm for acute pancreatitis. ICU: Intensive care unit; MOF: Multi-organ failure; FTT: Failure to thrive; ACS: Acute compartment syndrome; VARD: Video assisted retroperitoneal debridement.

conservative and minimally invasive approach. Eighty-eight patients were enrolled in the study, 44 underwent an open necrosectomy and 1 underwent VARD. In the step-up arm 43 patients were assigned to undergo minimally invasive approach according to a protocol. Specifically, the majority would begin with percutaneous drainage. If after 72 h of observation there were no documented clinical improvement, a second procedure would be pursued, commonly endoscopic drainage and/or VARD. The 35% of the patients assigned to the step-up approach, were treated with percutaneous drainage only. New onset multi-organ failure occurred less in the minimally invasive step-up approach group compared to the open necrosectomy (12% *vs* 40%). Although mortality was not significantly different between the two groups, long-term morbidity including new onset diabetes mellitus (16% *vs* 38%), incisional hernias (7% *vs* 24%) and pancreatic en-

zyme use (7% *vs* 33%) was higher in the open group and reached statistically significant difference in every parameter assessed. The same study group in 2011 published a prospective observational cohort study of 639 patients treated for pancreatic or peripancreatic necrosis. Sixty-two percent of the patients enrolled in the study were treated conservatively and 38% with an intervention (PCD, endoscopic transluminal catheter drainage, VARD, open necrosectomy). Mortality in the conservative group was 7% and 27% in the group undergoing intervention. Catheter drainage was the first intervention in 63% of cases and no additional necrosectomy was required in 35% of patients[15]. In choosing the correct approach to the necrotizing pancreatitis, an important aspect of management is timing and a randomized study[22] two retrospective studies[29,48] and a prospective study[15] clearly show a clinical benefit from postponing debridement for approximately 4 wk after admission. The use of less invasive techniques prior to that, if needed, will allow surgical debridement to be deferred or eventually avoided if possible[3]. Based on the above a treatment algorithm is proposed in Figure 1.

CONCLUSION

Most cases of acute pancreatitis are self-limited. However, necrotizing pancreatitis and more so infected necrosis, when they develop, can be associated with increased morbidity and mortality, making management a challenge. In the past, surgical necrosectomy through a laparotomy has been the mainstay for treatment of infected necrosis and cases of clinical deterioration despite maximal treatment. This approach is however associated with poor outcomes, is seldom used and should be considered as the last resort only. The management of infected necrosis has shifted towards less invasive approaches. It is now clearly recommended that a multidisciplinary group, when approaching a patient with severe acute pancreatitis complicated by necrosis and/or infection, should gear treatment towards the "3Ds" (Delay-Drain-Debride). Drainage early in the course of the disease, followed by endoscopic drainage, VARD or laparoscopy if debridement is necessary.

More randomized studies comparing a large number of this remarkably heterogeneous group of patients will further elucidate a more consistent protocol. The clinical features of each patient will currently dictate an individualized management plan made by experts in the appropriate setting, with the appropriate resources and equipment.

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2014 ADVANCES IN PANCREATITIS

New tools for optimizing fluid resuscitation in acute pancreatitis

Perrine Bortolotti, Fabienne Saulnier, Delphine Colling, Alban Redheuil, Sebastien Preau

Perrine Bortolotti, Fabienne Saulnier, Delphine Colling, Sebastien Preau, Critical Care Center, University Hospital of Lille, 59037 Lille, France

Perrine Bortolotti, Fabienne Saulnier, Delphine Colling, Sebastien Preau, Laboratoire de Physiologie, EA 4484, Lille Nord de France University, 59037 Lille, France

Alban Redheuil, Cardiovascular Imaging, Pitié Salpêtrière Hospital Institute of Cardiology, 75013 Paris, France

Alban Redheuil, Sorbonne Universités, Laboratoire d'Imagerie Biomédicale, 75005 Paris, France

Alban Redheuil, Université Pierre et Marie Curie-Paris 6, 75005 Paris, France

Alban Redheuil, ICAN Imaging Core Lab, 75005 Paris, France

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Correspondence to: Sebastien Preau, MD, PhD, Critical Care Center, University Hospital of Lille, Avenue du Professeur Emile Laine, 59037 Lille, France. seb.preau@gmail.com

Telephone: +33-20-444084 Fax: +33-20-445094
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Abstract

Acute pancreatitis (AP) is a frequent disease with degrees of increasing severity responsible for high morbidity. Despite continuous improvement in care, mortality remains significant. Because hypovolemia, together with microcirculatory dysfunction lead to poor outcome, fluid therapy remains a cornerstone of the supportive treatment. However, poor clinical evidence actually support the aggressive fluid therapy recommended in recent guidelines since available data are controversial. Fluid management remains unclear and leads to current heterogeneous practice. Different strategies may help to improve fluid resuscitation in AP. On one hand, integration of fluid therapy in a global hemodynamic resuscitation has been demonstrated to improve outcome in surgical or septic patients. Tailored fluid administration after early identification of patients with high-risk of poor outcome presenting inadequate tissue oxygenation is a major part of this strategy. On the other hand, new decision parameters have been developed recently to improve safety and efficiency of fluid therapy in critically ill patients. In this review, we propose a personalized strategy integrating these new concepts in the early fluid management of AP. This new approach paves the way to a wide range of clinical studies in the field of AP.

Core tip: Fluid therapy is a cornerstone of the early supportive treatment of acute pancreatitis. However, poor clinical evidence actually support the aggressive fluid therapy recommended in recent guidelines since available data are controversial. In this review, based on our experience of fluid management in the critically ill patients, we propose a tailored fluid administration relying on the individual benefit to risk balance, as a part of a global goal-directed hemodynamic strategy.

INTRODUCTION

The incidence of acute pancreatitis (AP), currently ranging from 13 to 45/100000 per year, increases steadily[1], making AP the first gastro-intestinal cause of hospi-

talization in the United States. Persistent organ failure occurring in the first few days is the main determinant of severity and defines severe AP[2]. Despite early management, in-hospital mortality of these patients, around 30%, remains high[3].

Due to numerous mechanisms, hypovolemia is a well-recognized risk factor of poor outcome in patients with AP[4]. During severe AP, an uncontrolled inflammatory response alters endothelial functions leading to vasodilation, capillary leakage and edema. Together with vomiting, ascite or ileus, this vascular dysfunction promotes hypovolemia and acute circulatory failure. Circulatory dysfunction leads to tissue hypoperfusion, ischemia and subsequently to self-sustaining disease with persistent pancreatic injury, extra-pancreatic tissue damage and organ failures[5].

Despite better knowledge of its pathophysiology[6,7], treatment of AP remains mostly supportive[8]. Rapid fluid perfusions, so called fluid loading or volume expansion are a cornerstone of AP management. Fluid loading allows rapid correction of hypovolemia, and efficient prevention of circulatory dysfunction[9]. Nevertheless, if appropriate fluid resuscitation prevents worsening of pancreas injury and development of organ failures, it may lead to poor outcome when excessive or insufficient[10-14]. Because of potential adverse effects, fluid resuscitation should therefore be cautiously administered in accordance with relevant evidence.

OPTIMIZING FLUID RESUSCITATION IN ACUTE PANCREATITIS: WHAT IS RECOMMENDED? WHAT IS CURRENTLY DONE?

When taking care of patients suffering from AP, it is strongly recommended to immediately assess hemodynamic status and begin resuscitative measures[15]. Early and aggressive fluid resuscitation is usually recommended and seems to reduce morbidity and mortality[1,15-19].

Early resuscitation refers mostly to fluid loading within the first 24 h of management[2,9,20]. Aggressive resuscitation is a liberal strategy of fluid administration to reach predetermined endpoints. In the latest guidelines, aggressive fluid therapy is defined as the administration of 250-500 mL per hour to all patients, except for those suffering from cardiovascular, renal and other comorbid conditions. Moreover, in case of suspicion of severe volume depletion, additional fluids are recommended. Proposed endpoints for guiding fluid therapy are mostly based on clinical parameters [arterial blood pressure, heart rate (HR) and urinary output (UO)], blood urea nitrogen (BUN)[3,15], hematocrit changes at 12-24 h after admission, and optionally central venous pressure (CVP)[4,9,21]. Finally, based on these endpoints, reassessment of fluid requirement is advised every 6 h within the first 24 to 48 h.

Nevertheless, there is poor consistent evidence to support such fluid strategy[5]. Recommendations are based on moderate levels of evidence, since studies are mostly observational with conflicting results[6,9]. As a result, current practice shows great heterogeneity, with various attitudes regarding fluid administration and chosen endpoints. In a recent New Zealand survey, physicians declared using aggressive fluid therapy in AP with organ failure. More than 70% of physicians estimated giving more than 4 L of fluids in patients with severe AP during the first 24 h after hospital admission. In theory, fluid administration as recommended might lead to an amount of about 6-12 L of fluids during the first 24 h[7,9,15]. However, aggressive fluid therapy as routinely performed corresponds to an average of 4.5 L of fluid over the first 24 h[8,9], against 3.5 L for non-aggressive therapy. In the same survey, fluid loading was mostly guided by UO, HR, blood pressure, hematocrit, BUN and lactate, even if the latter is not mentioned in the recommendations.

This explains the current controversy in the literature about necessary fluid volume, adequate timing and endpoints to achieve[5,8,9]. Moreover, some studies rather support restrictive strategies and report a positive impact on mortality[5,10,16]. Indeed, aggressive fluid loading may be detrimental, not only for patients suffering from AP[2,11,12,22] but more generally when any significant fluid therapy is needed[3,5-7,10,13-15,23-25]. The failure to clearly demonstrate the superiority of one fluid strategy over another may come from the great variability of individual response to volume expansion and the specific hemodynamic status of each patient at a given time. Consequently, aggressive therapy may be appropriate for some patients and deleterious for others.

New methods allowing better hemodynamic and fluid management have been developed over the last 15 years. These strategies aim to restore specific hemodynamic parameters with an individualized management named "early goal-directed therapy", in which fluid expansion takes a major part. The first step of this method is to clearly determine the specific population to which it should be applied. The second step is to assess tissue perfusion and oxygenation goals to be achieved. The last step is to choose the appropriate therapy in order to reach these predetermined goals. Fluid management then becomes part of a global hemodynamic strategy that has proved to be valuable in high-risk surgical patients and severe sepsis[16,26]. Understanding how hemodynamic criteria can be used to guide fluid therapy in these patients would help improving care and research in the field of AP[1,17,27].

GLOBAL HEMODYNAMIC RESUSCITATION: THE EARLY GOAL-DIRECTED THERAPY

Early goal-directed therapy (EGDT) is an aggressive, time-sensitive and individualized approach of global hemodynamic management. It is started within the very first hours after admission, before the occurrence of per-

Table 1 Diagnostic criteria for acute pancreatitis with high risk of poor outcome

Criteria for high risk of poor outcome	Hospitalization setting	Organ or system dysfunction
Severe AP: Persistent organ or system dysfunction (> 48 h)	Intensive care	Cardio-vascular: SAP < 90 mmHg despite 20-30 mL/kg fluid loading Respiratory: PaO_2 < 60 mmHg

Risk factors for severe AP:
  Organ or system dysfunction (< 48 h)
  Lactate > 3 mmol/L
  Persistent SIRS[1] (> 24 h)
  Pancreatic necrosis
  Pleural effusion or pulmonary infiltrates
  BUN > 20 mg/dL or rising BUN
  Hematocrit > 40% or rising hematocrit
  Age > 55 yr or comorbid disease or obesity
	Intermediate or intensive care	Renal: Creatinine $\geqslant$ 2 mg/dL or UO < 0.5 mL/kg of body weight/h for 1 h, despite 20-30 mL/kg fluid loading
		Hematological: Platelet count < 80000/mm³ or decrease > 50% of initial platelet count
		Metabolic: pH $\leqslant$ 7.30 or base deficit $\geqslant$ 5.0 mmol/L in association with lactate > 3 mmol/L Gastro-intestinal:
		  Gastro-intestinal bleeding (> 500 mL/24 h)
		Neurological: Altered mental status

[1]SIRS is defined by the presence of $\geqslant$ 3 of the following criteria: Pulse > 90 beats/min, Respirations > 20/min or $PaCO_2$ < 32 mmHg, Temperature > 38 ℃ or < 36 ℃, WBC count > 12000 or < 4000 cells /mm³ or > 10% immature neutrophils. AP: Acute pancreatitis; BUN: Blood urea nitrogen; $PaCO_2$: Partial pressure of carbon dioxide in arterial blood; PaO_2: Partial pressure of oxygen in arterial blood; SAP: Systolic arterial pressure; SIRS: Systemic inflammatory response syndrome; UO: Urinary output; WBC: White blood cell.

sistent organ failure that it aims to prevent[13,27].

This strategy arises from the finding that early aggressive therapy in acute diseases such as stroke, trauma or acute myocardial infarction improves mortality and outcomes[27]. EGDT has been conceived for optimizing treatment when tissue oxygenation is impaired by hemodynamic failure. It is a multifaceted strategy aiming to adjust oxygen delivery to oxygen consumption[13,14]. The concept of a global hemodynamic strategy guided by oxygen transport variables was first proposed in 1983 for high-risk surgical patients[28]. EGDT as a time-sensitive method has been initially applied to patients suffering from severe sepsis and septic shock[27], then in all patients with elevated lactate level, regardless of etiology[13]. It also has been proposed for perioperative management of patients undergoing major surgery, like cardiovascular or gastro-intestinal surgeries[29-33]. In these populations, EGDT is a now widely performed strategy that reduces morbidity, mortality and healthcare resource consumption[26,27,34]. Although no human trial evaluated such strategy in AP, most patients suffering from AP share similar pathophysiology, risk factors and severity with patients in whom this approach has been studied. Thus, even though clinical studies are needed to allow transposition to AP, EGDT may be suitable for this severe disease in the course of which many rapid hemodynamic changes can happen[35,36].

Immediate identification on admission of patients requiring EGDT based on the evaluation of the patient severity and potential outcome constitutes the very first step of the strategy. In severe sepsis and septic shock, EGDT is performed when patients present persistent hypotension with systolic blood pressure < 90 mmHg after a volume expansion of 20-30 mL/kg over a 30-min period or hyperlactatemia > 4 mmol/L[14]. In their study, Jansen *et al*[13] performed EGDT for every patient with lactatemia > 3 mmol/L on admission to the ICU. When included, patients were stratified into four groups: sepsis, neurologic, cardiac arrest and other nonsepsis, which accounted for 38% of the inclusions. Even though the au-

thors did not mention whether some AP were included, these patients frequently meet these inclusion criteria.

Twenty percent of patients will develop moderately severe to severe AP[37], characterized by the presence of either local or systemic complication, or organ failures. The resolution of organ failures in the first two days defines moderately severe AP. This group has prolonged hospitalizations and requires ICU care in 50% of cases, but maintains a mortality rate similar to the mild AP group[38]. Persistent organ failure is the main determinant of severity in AP and defines severe AP. Eighty percent of patients with severe AP will stay in the ICU. As patients with severe AP are at high risk of poor outcome, patients with high risk of severe AP would be considered at risk of poor outcome too. Despite the lack of reliable markers for early prediction of AP severity, several indices have been proposed[15]. Thus, along with refractory hypotension and elevated lactatemia, established risk factors for severe AP might be good candidates for early detection of patients at risk of poor outcome (Table 1). Nevertheless, further studies are needed to determine the most suitable parameters for early identification of at risk-patients in whom EGDT would be needed in this setting.

For those pre-selected patients, optimization of parameters reflecting tissue perfusion and oxygenation remains the major goal to achieve during severe sepsis and high-risk surgery. Thus, essential determinants or estimates of oxygen delivery are assessed step by step and corrected if needed.

In order to monitor and optimize microcirculatory function, HR and mean arterial pressure (MAP) are mainly used. As tachycardia remains a clinical sign of circulatory failure therapeutic strategy aims to lower HR under 100 beats/min. MAP, reflecting effective organ perfusion pressure, has to be maintained above 65 mmHg[27].

Microcirculatory function, finally ensuring tissue perfusion, can be estimated by lactate level and UO[27,39,40]. Lactate level increases when aerobic cellular respiration is impaired and switched towards anaerobic metabolism.

UO, in roughly reflecting glomerular perfusion, provides valuable information on general tissue perfusion. Both are good clues to evaluate tissue perfusion even if not entirely specific. For instance, lactate levels can possibly increase in rare metabolic diseases or when liver failure occurs. UO can be altered during organic renal failure, independently of hemodynamic disorders[41]. Similarly, mottling score, reflecting skin hypoperfusion can also be helpful to estimate global tissue perfusion[42,43]. EGDT aims to normalize lactate level and Jansen and al. targeted a 20%-decrease every two hours[13]. Therapeutic intervention also aims to maintain UO over 0.5 mL/kg per hour and make mottling disappear.

The balance between oxygen delivery (DO_2) and systemic oxygen consumption (VO_2) is approached by measurement of central venous oxygen saturation ($ScvO_2$). Its measurement can be easily performed on a blood sample taken from a central venous catheter inserted in the superior vena cava territory. SvO_2 depends on global oxygen transport and tissue oxygen extraction and consumption as can be seen in the modified Fick equation: $SvO_2 \approx SaO_2 - [VO_2/(CO \times Hb \times 1.34)]$ where SaO_2 represents arterial oxygen saturation, CO cardiac output and Hb hemoglobin[44]. Each parameter described previously should be optimized to reach an $ScvO_2$ level > 70%, associated with a normal lactate level. Importantly, when $ScvO_2$ is superior to 70% but lactate level remains high, the presence of microcirculatory dysfunction with oxygen extraction impairment leading to persistent tissue hypoxia despite adequate oxygen transport should be suspected.

To carry out this step-by-step strategy, patients should be closely monitored. Together with standard monitoring of vital signs, specific devices including central venous catheters and urinary catheters have to be implemented when patients meet severity criteria. EGDT is then implemented during the first 6-8 h of the patient's management. Previously described endpoints should be closely and regularly checked to assess treatment efficiency. For instance, Rivers *et al*[14] checked endpoints every 30 min. Jansen *et al*[13] measured blood lactate level together with other chosen endpoints every two hours.

Global hemodynamic goals are achieved by numerous treatments (*e.g.*, fluids, red blood cell transfusion, oxygen, ventilation, analgesics, sedatives, antipyretics, vasoconstrictors, vasodilators and cardiac treatments) depending on the presence of hypovolemia, anemia, low SaO_2, vasoplegia and cardiac dysfunction. In this global approach, fluid therapy plays an early and major role (Figure 1). A rigorous management of fluid loading is essential to succeed in reaching endpoints and requires simple but adequate guiding tools.

MANAGEMENT OF FLUID RESUSCITATION: A CORNERSTONE OF THE EARLY GOAL-DIRECTED THERAPY

The clinician's major concerns are to assess for each fluid prescription whether fluid infusion would improve the patient's hemodynamics and organ perfusion, with minimal risk of adverse effect. Three situations can be encountered. The first one is a patient with undisputed need for volume expansion, presenting obvious hypovolemia with a clearly identified etiology. For instance patients with severe AP or sepsis at the very beginning of the treatment are very likely hypovolemic and usually receive 20-30 mL/kg of fluids within the first 60-90 min. In this case, the benefit to risk balance is obvious. The second situation is obvious fluid overload such as a patient with congestive heart failure and acute pulmonary edema for whom volume expansion would clearly be deleterious. The last situation concerns patients with hemodynamic impairment for whom volume expansion represents a major therapeutic option, but with uncertain benefit to risk balance. This remains the most frequently encountered case for which specific tools have been created. Indeed, when only based on clinical parameters (*e.g.*, mottling, HR, blood pressure or UO), barely one half of the critically ill patients will respond positively to fluid loading[45]. Because of the potential adverse effects of inappropriate fluid perfusions[10-14,46], tools intended to assess and predict the effects of fluid loading may be helpful to guide fluid therapy and improve patients outcome.

When applied in practice, EGDT leads to differences in patients' fluid management. Rivers and al. found that a greater amount of fluid was given to the EGDT group compared with the standard group in the first 6 h (4981 mL *vs* 3499 mL; $P < 0.001$), even though the total amount of fluid over the first 72 h was similar (13443 mL *vs* 13358 mL; $P = 0.73$). As a result, a 30% decrease in hematocrit associated with a larger amount of transfusion of red blood cells was observed in the EGDT group compared with standard care in the first 6 h[27]. As the beneficial effect of this strategy is based on the adaptation of hemodynamic management on tissue oxygenation, there is still a lack of evidence concerning the best tools to use for guiding fluid resuscitation.

Assessing fluid responsiveness: fluid challenge
Fluid challenge (FC) intends to assess a patient's fluid responsiveness during a volume expansion test. First described by Weil and Henning[47], FC is a titrated administration of 50-200 mL of fluid over a 10 min interval, with a concomitant close monitoring of patient's cardiovascular response. Fluid responsiveness is defined by a fluid-induced increase in stroke volume (SV), or in CO as the product of SV by HR. A positive response is considered when fluid loading leads to an increase in SV $\geq$ 10%-15%[45]. Indeed, if optimization of systemic hemodynamics and tissue perfusion remains the ultimate goal of fluid therapy, increase in SV is considered as a prerequisite to achieve it[48]. FC is the reference standard method to distinguish responders from non-responders to fluid loading[34]. Current international guidelines recommend 250-1000 mL of crystalloids or 250-500 mL of colloids over 15-30 min, repeated after reassessment until endpoints are achieved[26,34,49].
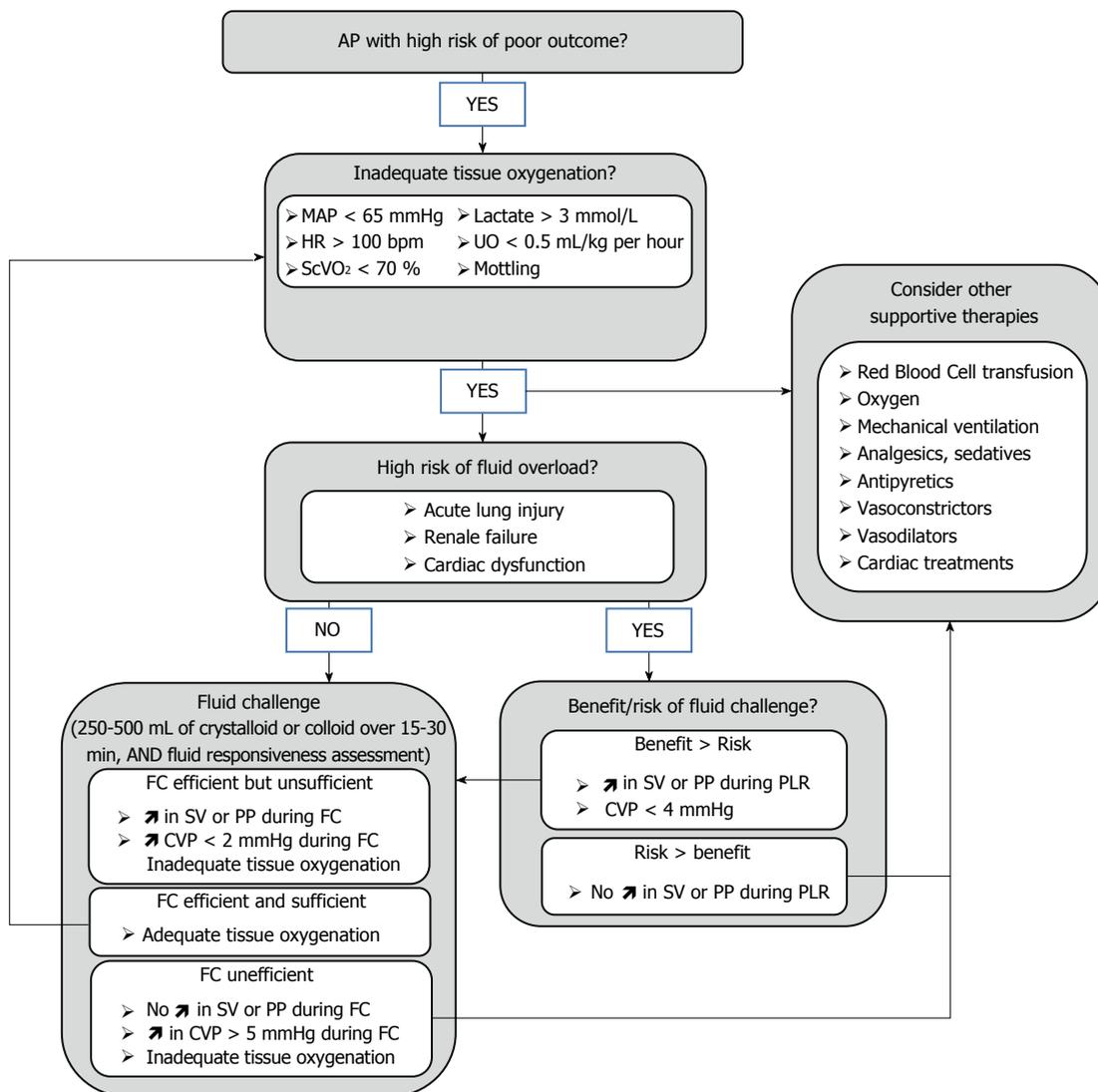
Figure 1 Suggested algorithm for fluid management in acute pancreatitis. AP: Acute pancreatitis; MAP: Mean arterial pressure; HR: Heart rate; ScvO2: Central venous oxygen saturation; UO: Urinary output; SV: Stroke volume; PP: Arterial pulse pressure; PLR: Passive leg raising; CVP: Central venous pressure; FC: Fluid challenge.

When performed in anesthesiology, where invasive monitoring techniques such as trans-esophageal Doppler, esophageal echocardiography or thermodilution enable continuous assessment of CO, fluid infusion is continued as long as CO increases[50,51]. However, continuous CO measurement is often not available for non-surgical patients. In that case, noninvasive measurement of SV before and after FC with transthoracic echocardiography is a relevant parameter to estimate fluid responsiveness[52].

If SV monitoring cannot be performed, blood pressure derived indexes may help to predict fluid responsiveness. Indeed, fluid-induced changes in arterial pulse pressure (PP) are correlated to some extent to changes in SV[53,54]. Monnet *et al*[54] found that a fluid-induced increase in invasive PP over 17% attested of fluid responsiveness with a sensitivity of 65% and a specificity of 85%. Lakhal *et al*[53] showed that an increase beyond 23% for invasive PP, or 35% for noninvasive PP reliably predicted fluid responsiveness. On the opposite, fluid responsiveness was

unlikely under 5% of PP change. Nonetheless, the large range of inconclusive results (*i.e.,* 5%-17% of changes in PP) represents a major limit of this method.

In parallel, dynamic analysis of CVP can be monitored as an indicator of safety limits[13,47,55]. CVP is commonly used as an estimation of cardiac preload at the bedside. Preload is defined as the load in cardiac chambers present before isovolumetric ventricular contraction has started. It represents the stress exerted on ventricular walls in end diastole. Venous return is a major determinant of preload and is mostly dependent on volemia. Thus, hypovolemia decreases preload whereas volume expansion increases it. Described by Frank and Starling, there is up to a certain limit a positive relationship between end-diastolic ventricular load and systolic SV, called preload-dependence[45]. In that case, fluid administration leads to a large increase in SV while CVP remains stable or presents only a minimal increase. Preload-dependence is thus associated with a positive response to volume ex-
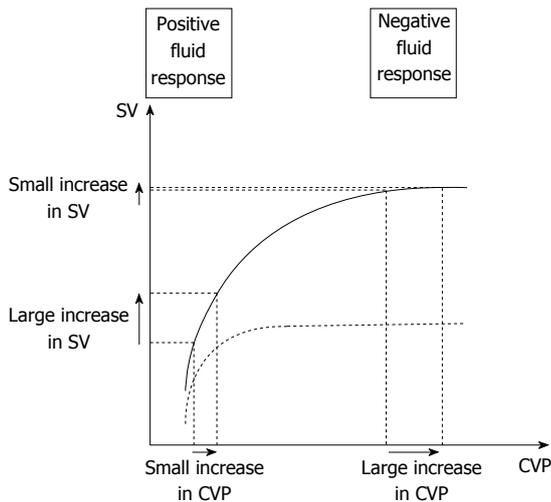
Figure 2 Schematic representation of central venous pressure/stroke volume of normal (solid line) and failing heart (dotted line). When the heart is fluid responsive, a fluid challenge induces a large increase in stroke volume (SV) and a small increase in central venous pressure (CVP). When the heart is fluid unresponsive, a fluid challenge induces a small increase in SV and a large increase in CVP. In contrast, there is no reliable threshold of CVP that can be used in current practice to predict a positive or negative response to fluid loading. This threshold depends mostly on the cardiac function at the time of fluid infusion.

pansion. However, beyond a certain individual threshold, an increase in preload does not increase SV anymore, which corresponds to a preload-independence state. For those patients, fluid administration leads to poor SV improvement but consistent increase in CVP with high risk of fluid overload (Figure 2). Subsequently, volume expansion-induced changes in CVP have been proposed as a safety limit of FC[47,55]. As long as changes in CVP remain below 2 mmHg FC is continued until hemodynamic endpoints are fulfilled. For an increase in CVP ranging from 2-5 mmHg, fluid infusion should be stopped for a while then restarted. Over a 5 mmHg increase, FC should be stopped. The time interval to assess filling pressures and fluid responsiveness was every 10 min in the initial description. However, with the availability of continuous vital signs monitoring, the intervals may be extended to 30 min.

FC allows a prompt correction of fluid deficit, with a shorter duration of hypovolemia and organ hypoperfusion, compared with a protracted fluid infusion strategy over 12 h or more[55]. FC only requires a central venous catheter to control safety limits, together with conventional monitoring of vital signs and CO if available (Figure 1). Nevertheless, this strategy, although approved by experts and routinely used in intensive care has never been confirmed by a prospective controlled trial[55]. In addition, despite close monitoring, the effect of fluid infusion is retrospectively assessed, and the repetition of FC might lead to fluid overload. Such risk remains a major concern for patients with AP, as they present an increased risk of acute lung injury[56]. Therefore, fluid responsiveness should ideally be estimated before fluid is administered to avoid ineffective or deleterious fluid administration for patients

with unclear benefit to risk balance, such as those who develop pulmonary, cardiac or renal dysfunction[11,12,57]. New parameters aiming to predict fluid responsiveness have been developed to this end.

Predicting fluid-responsiveness: preload and preload-dependence

The ultimate goal of tools aiming to predict fluid-responsiveness is to find where individual ventricular hemodynamic status is located on the Franck-Starling curve (Figure 2). In other terms, indexes predicting fluid responsiveness are assessing cardiac preload-dependence[58].

Based on aforementioned physiological concepts, one could postulate that low preload values are more likely to be associated with preload-dependence and conversely for high preload values. However, several studies show that this assertion is not true. When CVP or pulmonary artery occlusion pressure (PAOP) are used as estimates of cardiac preload, they usually fail to predict fluid responsiveness[45,59]. This can easily be understood because Franck-Starling curve is specific to each patient[45]. Thus, there is no way to know whether a single absolute CVP or PAOP level corresponds to a preload-dependence or -independence zone[60] (Figure 2). Even for extreme values of CVP or PAOP, there is no reliable threshold that can be used in current practice to predict a positive or negative response to volume expansion[45,59]. However, preload evaluation, and particularly CVP measurements are still recommended in hemodynamic management algorithms for several reasons[34]. First, it is easy to assess, only requiring a central venous catheter. Second, as detailed above dynamic analysis of CVP is still valuable in evaluating FC response. Eventually, CVP values standing below 4 mmHg, even if not predictive of fluid responsiveness, ensure safe fluid loading with little risk of overfilling[34,61] (Figure 1).

Consequently, indexes predicting fluid-responsiveness focus on preload-dependence rather than preload assessment[58]. Passive leg raising (PLR) maneuver is an easy maneuver that mimics volume expansion by shifting venous blood from the lower limbs and the splanchnic vessels toward the intrathoracic vessels[62]. Thus, PLR leads to a rapid and reversible increase in cardiac preload and subsequently in SV in case of preload dependence. To be efficient, PLR maneuver has to be performed as follows[63]: the patient's baseline position is lying down on a bed, half-sitting in semirecumbent position, with a 45° angle between trunk and lower limbs, which are horizontal. Then, a 45° bascule of the bed should be done, so that the trunk becomes horizontal and the lower limbs rise up. Impact of the maneuver appears within the first minute, while the hemodynamic measurements are recorded. PLR mimics an approximate 300-450 mL FC[63,64]. A close correlation is observed between changes in SV measured with TTE or esophageal Doppler, after PLR and after a 500 mL of fluid loading in critically ill patients with sepsis or acute pancreatitis[64-67]. When considering a recent meta-analysis enrolling 9 clinical studies that evalu-

ated the accuracy of PLR to predict fluid responsiveness, a PLR-induced change in SV superior to 8%-15% predicted fluid responsiveness with a sensitivity of 89% and specificity of 91%[68]. When considering PP as a surrogate of SV, a PLR-induced change in PP > 9%-12% predicts fluid responsiveness with sensitivity of 60% and a specificity of 86%[68] (Figure 1). The main limit of the PLR technique is the presence of intra-abdominal hypertension. Indeed, in ventilated critically ill patients, Mahjoub *et al*[69] showed that PLR failed to predict fluid responsiveness when intra-abdominal pressure exceeded 16 mmHg due to false negatives. As demonstrated by Kitano *et al*[70], Takata *et al*[71] when intra-abdominal pressure exceeds right atrial pressure, the inferior vena cava collapses and impairs venous return. The PLR-induced change in cardiac preload is decreased making the PLR maneuver inefficient[69-71]. As intra-abdominal hypertension is a common complication of AP, intra-abdominal pressure should be measured before using PLR.

Other indexes based on heart-lung interactions have also been developed. However, they are only validated for mechanically ventilated patients under strict conditions of sedation, ventilation and cardiac rhythm[72]. Because the proportion of patients requiring mechanical ventilation during AP remains very low, with specific multidisciplinary management in intensive care[56], these parameters are not discussed in this review. In spontaneously breathing patients, respiratory variations in inferior vena cava diameter or PP are still in development[73-75]. As existing data were not confirmed in large population studies, and as most of them didn't include patients with AP, the use of such parameters in spontaneously breathing patients with AP seems hazardous and yet to be validated.

The impact of fluid therapy based on preload-dependence parameters has been evaluated in studies involving surgical patients[76-78]. When compared with liberal or preload-based fluid administration, the use of preload-dependence parameters drives to a decrease in lactate level, perioperative complications and time to discharge. Interestingly, this strategy leads either to a greater[77,78] or to a lesser[76] amount of fluid compared with the control group. These results suggest that the efficiency of such strategy comes from volume expansion adjustment to patient's needs rather than from the total amount of fluid administered. Patients involved in these studies were mechanically ventilated and no similar trial exists in spontaneously breathing patients. Moreover, there is a great lack of data in patients with AP. Nevertheless, a recent study performed on anesthetized pigs with experimental AP compared a fluid therapy based on preload-dependence indexes to a CVP-based strategy. The fluid therapy guided by preload-dependence parameters increased survival (29.4% *vs* 11.8%; *P* < 0.05) by preventing microcirculation dysfunction, pancreatic damages and pulmonary edema. These results are concordant with human findings described just before, and confirm the inability of CVP to guide fluid therapy[79]. These encouraging data might open the way to further research in humans with AP.

CONCLUSION

Adopting an individualized early goal-directed strategy seems very promising to optimize fluid resuscitation in patients with AP. However, since AP has specific pathophysiology, evolution, complications and outcome, further studies are required to provide a suitable algorithm. The first step will be to define parameters allowing early identification of patients needing EGDT, notably those at risk to develop severe or necrotizing AP. Among parameters previously described in the literature, elevated lactate level and refractory hypotension could be good candidates. The second step is to clearly define ultimate goals of hemodynamic resuscitation reflecting tissue perfusion and oxygenation. If those are not achieved, EGDT should immediately be implemented and carried on until adequate systemic perfusion is restored. Close reassessment of initial endpoints has to be performed every 30 min to readjust treatment without delay. Because volume expansion plays a major role in this strategy, fluids should be administered early. Inadequate fluid replacement can occur when guided on clinical parameters alone, static preload assessment with CVP or worse, blindly. A safe and practical way to perform fluid loading remains FC, with simultaneous assessment of fluid-responsiveness and control for risk of overload. However, for patients with high risk of fluid overload, predicting fluid-responsiveness before volume expansion may reduce the number of FC and improve patient outcomes. PLR is an accurate validated maneuver to predict fluid-responsiveness. It can widely be used provided the absence of intra-abdominal hypertension. These considerations open the way to a wide range of clinical studies aiming to adapt and validate such strategies in the specific population of patients with AP.

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2014 ADVANCES IN PANCREATITIS

Enteral nutrition in acute pancreatitis: A review of the current evidence

Attila Oláh, Laszlo Romics Jr

Attila Oláh, Department of Surgery, Petz Aladár Teaching Hospital, H-9023 Győr, Hungary

Laszlo Romics Jr, Department of Surgery, Glasgow Victoria Infirmary, Glasgow G42 9TY, United Kingdom

Author contributions: Oláh A designed research; Oláh A and Romics Jr L performed literature search, analyzed data and wrote the paper.

Correspondence to: Attila Oláh, MD, PhD, MRCS, Department of Surgery, Petz Aladár Teaching Hospital, P.O. Box 92, H-9023 Győr, Hungary. olaha@petz.gyor.hu

Telephone: +36-96-418244 **Fax:** +36-96-507907

Abstract

The use of enteral feeding as part of the management of acute pancreatitis dates back almost two decades. This review describes the indications for and limitations of enteral feeding for the treatment of acute pancreatitis using up-to-date evidence-based data. A systematic review was carried out to analyse current data on the use of enteral nutrition in the management of acute pancreatitis. Relevant literature was analysed from the viewpoints of enteral *vs* parenteral feeding, early *vs* delayed enteral nutrition, nasogastric *vs* nasojejunal feeding, and early oral diet and immunonutrition, particularly glutamine and probiotic supplementation. Finally, current applicable guidelines and the effects of these guidelines on clinical practice are discussed. The latest meta-analyses suggest that enteral nutrition significantly reduces the mortality rate of severe acute pancreatitis compared to parenteral feeding. To maintain gut barrier function and prevent early bacterial translocation, enteral feeding should be commenced within the first 24 h of hospital admission. Also, the safety of nasogastric feeding, which eases the administration of enteral nutrients in the clinical setting, is likely equal to nasojejunal feeding. Furthermore, an early low-fat oral diet is potentially beneficial in patients with mild pancreatitis. Despite the initial encouraging results, the current evidence does not support the use of immunoenhanced nutrients or probiotics in patients with acute pancreatitis.

Core tip: The application of enteral feeding in acute pancreatitis is much debated. This systematic review provides global insight for clinicians on how to incorporate enteral feeding in the management of acute pancreatitis. The timing, route and composition of enteral nutrition are discussed with up-to-date evidence-based data, and the latest relevant guidelines are also detailed. Importantly, enteral nutrition significantly reduces mortality in severe acute pancreatitis compared to parenteral nutrition. Furthermore, early commencement of enteral feeding (within the first 24 h) is beneficial, and the safety of the nasogastric route seems to be equal to that of the nasojejunal route.

INTRODUCTION

The treatment of acute pancreatitis is purely symptomatic because there is no effective therapy to prevent the activation of inflammatory and proteolytic cascades. This vicious cycle of cell signalling is believed to be triggered by bacterial infection, predominately Gram-negative strains.

The most likely hypothetical source of the bacterial infection is the gastrointestinal tract. Bacterial translocation is caused by increased permeability in the gut and a consequent migration of macromolecules such as bacteria, endotoxins and antigens from the gastrointestinal tract to the portal system, mesenteric lymph nodes, liver, spleen and pancreas. This process leads to the stimulation of macrophages, circulatory neutrophils and granulocytes, and then the release of pro-inflammatory cytokine causes an inflammatory response. If the inflammatory response, which is initially part of the defence mechanisms of the host, is over-activated, it may turn into a self-destructive process. The unbalanced production of inflammatory mediators might lead to the development of systemic inflammatory response syndrome (SIRS), infectious pancreatic necrosis and ultimately multi-organ failure (MOF)[1].

Severe acute pancreatitis (SAP) represents a typical model of septic syndrome due to a failure of the gut barrier. Hence, one of the main therapeutic goals in SAP is to maintain gut integrity to prevent bacterial and endotoxin translocation and improve the immune system of the gut. Recently, various clinical methods have aimed to prevent or decrease bacterial translocation. These methods include enteral feeding, with or without immunonutrition, as well as the use of probiotics during treatment. This paper provides a review of the available data in the evidence-based literature on the application of enteral feeding to acute pancreatitis. Furthermore, the authors discuss the evidence supporting early as opposed to delayed commencement of enteral feeding. The latest results on immunonutrition and probiotic use are also presented. In addition, the debate on the adequate route for enteral feeding is outlined. Finally, up-to-date guidelines and clinical practices are discussed.

ENTERAL NUTRITION *VS* PARENTERAL NUTRITION

Prolonged parenteral feeding carries numerous unfavourable side-effects such as atrophy and increased permeability of the gut mucosa. Furthermore, the lack of peristaltic stimulation results in hypomotility of the gut, and the stagnant bowel contents also cause significant changes in the intestinal microflora. Conversely, enteral feeding prevents the aforementioned atrophic changes as the uptake of nutrients in intestinal epithelial cells comes directly from the intestinal lumen. In addition, enteral feeding facilitates gut motility due the hyperosmolarity of the nutrients. These pathophysiological mechanisms protect against the overgrowth of abnormal intestinal flora and increased gut permeability, hence, potentially alleviating subsequent bacterial translocation.

In the first two randomised prospective trials, Mc-Clave *et al*[2] and Nakad *et al*[3] demonstrated that nasojejunal feeding is feasible, safe and beneficial in mild to moderate pancreatitis, or even severe pancreatitis. Altogether, 16 randomised controlled trials involving 847 patients

with acute pancreatitis compared enteral to parenteral feeding[2,4-18]. Eleven of these studies randomised patients with severe pancreatitis or predicted SAP. All meta-analyses demonstrated a statistically significant reduction of infectious complications with the use of enteral nutrition[19-21], except two studies which failed to confirm the beneficial effect of nasojejunal feeding[6,16]. Hence, enteral nutrition has been established as a key component in the management of SAP[22].

The meta-analyses published by Marik and Zaloga[21] as well as McClave *et al*[23] demonstrated that the use of enteral nutrition resulted in a significant reduction of infectious complications and length of hospital stay, as well as a trend toward reduced organ failure. However, these meta-analyses failed to confirm that enteral feeding could reduce mortality. In a meta-analysis published by Petrov *et al*[20], more homogenous subgroups were compared, and altogether 202 patients with predicted SAP were included. The mortality rate in the enteral nutrition group was 4% (4/95) *vs* 15.9% (17/107) in the parenteral nutrition group, a statistically significant difference (RR = 0.32; 95%CI: 0.11-0.98; *P* = 0.03). Furthermore, Cao *et al*[19] analysed six randomised controlled trials involving 224 patients and demonstrated a statistically significant decrease in mortality (OR = 0.251; 95%CI: 0.095-0.666, *P* = 0.005) and MOF (OR = 0.306; 95%CI: 0.128-10.736) in patients receiving enteral feeding. Finally, in a recent meta-analysis involving 381 patients from eight randomised controlled trials, Yi *et al*[24] found that total enteral nutrition is significantly superior to total parenteral nutrition in patients with SAP. A statistically significant difference was observed in mortality (*P* = 0.001; OR = 0.37; 95%CI: 0.21-0.68), infectious complications (*P* = 0.004; OR = 0.46; 95%CI: 0.27-0.78), organ failure (*P* = 0.02; OR = 0.44; 95%CI: 0.22-0.88) and surgical intervention (*P* = 0.003; OR = 0.41; 95%CI: 0.23-0.74). However, no difference was detected in terms of length of hospital stay and duration of nutrition administration.

In summary, the data accumulated so far provide strong evidence of the benefits of enteral over parenteral nutrition in patients with SAP, because the risk of mortality was statistically less in patients given enteral compared to parenteral nutrition. Importantly, in the clinical setting, there are no specific contraindications for enteral nutrition. It can be performed safely even when SAP is complicated by fistulas, ascites or pseudocysts. The role of parenteral nutrition is limited in conditions such as severe ileus, when enteral nutrition can be restricted by paralysis. Nevertheless, enteral feeding in reduced amounts is still suggested in these cases, which provides the physiological benefits discussed above[25] (Table 1).

EARLY *VS* DELAYED ENTERAL NUTRITION

Although the exact pathophysiological mechanisms of bacterial infection have not been determined, it seems unequivocal that it is a significant risk factor for pancre-

Table 1 Studies investigating the potential benefits of enteral *vs* parenteral feeding

Ref.	Year	Country/institution	No. of patients	Control arm	Benefits of enteral *vs* parenterial feeding
McClave *et al*[2]	1997	United States/University of Louisville, KY	30	Parenteral feeding	Cheaper, better glucose control
Kalfarentzos *et al*[4]	1997	Greece/University of Patras	38	Parenteral feeding	Lower complication rate, cheaper
Windsor *et al*[5]	1998	United Kingdom/St James's Univ Hospital London	34	Parenteral feeding	Decreased organ failure and complication rates
Paraskeva *et al*[7]	2001	Greece/Pireus General Hospital	23	Parenteral feeding	Lower surgical intervention rate
Oláh *et al*[59]	2002	Hungary/Petz A. Teaching Hospital, Gyor	89	Parenteral feeding	Less septic complications
Abou-Assi *et al*[9]	2002	United States/Virginia Univ. Hosp., RA	53	Parenteral feeding	Less septic complications, cheaper
Gupta *et al*[10]	2003	United Kingdom/Southampton General Hospital	17	Parenteral feeding	Shorter hospital stay, cheaper
Louie *et al*[12]	2005	Canada/University of Alberta	28	Parenteral feeding	Lower complication rate, better glucose control
Eckerwall *et al*[37]	2006	Sweden/Lund University Hospital	69	Parenteral feeding	Lower complication, MOF and mortality rates
Petrov *et al*[13]	2006	Russia/Nizhny Novgorod Hosp	22	Parenteral feeding	No significant difference

MOF: Multi-organ failure.

atic necrosis and the development of MOF during SAP. Importantly, bacterial translocation and pathogen overgrowth can be detected in the very early phase of acute pancreatitis. In a multicentre study, Besselink *et al*[26] demonstrated that bacteraemia can be detected as early as day 7 and that infected necrosis can be detected on average 26 d after hospital admission. Furthermore, early bacterial invasion may aggravate SIRS, which in turn makes the patient even more susceptible to organ failure. This can result in a vicious cycle, because the development of organ failure frequently precedes bacterial infection. Hence, if bacterial translocation can be reduced or prevented through the maintenance of the intestinal barrier with enteral feeding, then it is reasonable to begin enteral feeding as early as possible.

A systematic meta-analysis published by Petrov *et al*[27] involving 11 randomised controlled trials demonstrated that the risk of MOF, pancreatic infectious complications and mortality were significantly reduced in patients with acute pancreatitis who were enterally fed within the first 48 h of admission as opposed to parenteral feeding. Importantly, the differences were not statistically significant, if enteral nutrition was commenced 48 h after admission. In fact, a large amount of evidence-based data support the administration of enteral nutrition within 24 h of hospital admission[28].

This has been further confirmed by Sun *et al*[29] in a recently published randomised controlled trial. The authors investigated the effects of early administration of enteral nutrition on the immune function and clinical outcomes of 60 patients with SAP. The incidences of multiple organ dysfunction syndrome, SIRS and pancreatic infection, as well as the duration of stay in the intensive care unit, were significantly lower in the early administration group (commenced within 48 h of hospital admission) than in patients whose enteral feeding began on the eighth day of hospital stay. However, the authors did not report a difference in mortality between the two groups, which could have been due to the rela-

tively low number of patients in the study. In another recent randomised controlled trial involving 197 patients with SAP, Wereszczynska-Siemiatkowska *et al*[30] found that enteral feeding within 48 h of admission significantly decreased the incidence of infective necrosis/fluid collection, respiratory failure, intensive care treatment and mortality compared to enteral feeding started after 48 h of hospital admission. While a clear trend towards a reduction in the rate of multi-organ dysfunction and surgical interventions was observed in patients with early enteral feeding, these differences were not statistically significant. An interesting aspect of the pathophysiology of acute pancreatitis was investigated in a pilot study by Sun *et al*[31], which compared the incidence of intra-abdominal hypertension in 60 patients with early (within 48 h) or delayed (after day 8) administration of enteral nutrition. Intra-abdominal hypertension was more prevalent in patients with delayed administration of enteral nutrition. They also argued that higher intra-abdominal pressure (over 15 mmHg) may correlate with intolerance to feeding (Table 2).

The above findings were confirmed by a recent meta-analysis on the benefits of early administration of enteral nutrition commenced within 48 h of hospital admission[32]. Based on 11 studies involving 775 patients, Li *et al*[32] concluded that early enteral feeding was associated with significant reductions in all infections (OR = 0.38; 95%CI: 0.21-0.68; $P < 0.05$), catheter-related septic complications (OR = 0.26; 95%CI: 0.11-0.58; $P < 0.05$), pancreatic infection (OR = 0.49; 95%CI: 0.31-0.78; $P < 0.05$), hyperglycaemia (OR = 0.24; 95%CI: 0.11-0.52; $P < 0.05$), length of hospitalisation [mean difference -2.18; 95%CI: -3.48-(-0.87); $P < 0.05$] and mortality (OR = 0.31; 95%CI: 0.14-0.71; $P < 0.05$). Importantly, a multi-centre randomised controlled trial that investigated 208 patients with predicted SAP has yet to report its results. The PYTHON trial, which was organised by the Dutch Pancreatitis Study Group, compared very early nasojejunal feeding (within 24 h of hospital admission) to standard

Table 2 Studies investigating the potential benefits of early *vs* late enteral feeding

Ref.	Year	Country/Institution	No. of patients	Control arm	Benefits of early *vs* late enteral feeding
Sun *et al*[29]	2013	China/Nanjing Medical University	60	Late enteral feeding	Lower infective complication, MOF and SIRS rates
Wereszczynska-Siemiatkowska *et al*[30]	2013	Poland/Medical University Bialystok	197	Late enteral feeding	Lower complication and mortality rates
Sun *et al*[31]	2013	China/Nanjing Medical University	60	Late enteral feeding	Lower intra-abdominal hypertension rate

MOF: Multi-organ failure; SIRS: Systemic inflammatory response syndrome.

practice (oral nutrition on demand, or if needed, enteral feeding after 72 h)[33].

NASOGASTRIC *VS* NASOJEJUNAL FEEDING

While the placement of a nasogastric tube is a simple routine procedure that can facilitate the commencement of early enteral feeding, nasojejunal feeding requires an endoscopist or radiologist for tube placement, which may cause a delay in the start of early enteral feeding. Hence, nasogastric feeding seems to be the most feasible option in clinical practice. However, arguments against nasogastric feeding are based on the effects of stimulating pancreatic secretion and gastric emptying problems due to paralysis.

Eatock *et al*[34] were the first to investigate these concerns in a prospective pilot study and found that nasogastric feeding is safe and well-tolerated. Then, two randomised controlled trials that compared nasogastric and nasojejunal feeding[35,36] concluded that nasogastric nutrition at a slow rate of infusion was well tolerated, and there were no differences in the outcome measures (discharge, surgery and mortality rate) between the two groups. Another randomised controlled trial, which compared early nasogastric feeding to total parenteral nutrition in patients with predicted SAP[37], demonstrated that enterally fed patients had significantly more total complications and pulmonary complications within the first 3 d. Two meta-analyses based on the above studies involving a total of 131 patients[38,39] revealed no significant differences in mortality rate, length of hospital stay, infectious complications or MOF in SAP between nasogastric enteric feeding and conventional feeding. A recent meta-analysis based on three randomised trials involving 157 patients drew the same conclusion, that nasogastric feeding is not inferior to nasojejunal feeding[40]. Although nasogastric feeding seems safe and well tolerated compared to nasojejunal feeding, more high-quality randomised controlled trials are needed to provide strong evidence, because the sample sizes in the studies conducted to date have been relatively low.

EARLY ORAL DIET

Regarding early oral feeding, a pilot study was the first to demonstrate the feasibility of administration of an oral diet an average of 3 d after hospital admission[41]. To determine if oral feeding is feasible for treating mild pancreatitis, Eckerwall *et al*[42] randomised 60 patients with mild acute pancreatitis to compare the efficacy and feasibility of immediate oral feeding and traditional fasting. No differences were found in amylase values or the systemic inflammatory response between the two groups. This trial proved that immediate oral feeding is feasible and safe for treating mild acute pancreatitis. Furthermore, two randomised controlled trials demonstrated that it is not necessary to keep the patient on a liquid diet after acute mild pancreatitis[43,44], as no detrimental effects were observed from a solid diet. In fact, a solid diet was associated with a shorter length of hospital stay[44]. Hence, patients with mild acute pancreatitis can be started on a low-fat oral diet, although an initial period of fasting is still reasonable[28].

IMMUNONUTRITION: GLUTAMINE SUPPLEMENTATION

Immunoenhanced nutrients involve substrates that modulate the activity of the host immune system and inflammatory response (Table 3). Immunonutrition formulas include glutamine, arginine, nucleotides and omega-3 fatty acids, as well as enteral nutrients supplemented by probiotics. Experimental studies have suggested that supplementation of enteral feed with glutamine or omega-3 fatty acids may reduce the severity of experimental acute pancreatitis[45]. However, results have been rather moderate in the clinical setting. The four randomised controlled trials on this subject[46-49] demonstrated that immunonutrition has some beneficial effects, such as a shortened length of hospital stay, reduced gut permeability and decreased plasma endotoxin levels, but no significant differences were found in terms of clinical outcomes. Furthermore, a meta-analysis published by Petrov *et al*[50] based on three randomised controlled trials[48-50] clearly demonstrated that immunonutrition, compared to standard enteral nutrition, was not associated with a significantly reduced risk of total infectious complications (RR = 0.82; 95%CI: 0.44-1.53; P = 0.53) or mortality (RR = 0.64; 95%CI: 0.20-2.07; P = 0.46).

As for glutamine supplementation, emerging evidence suggests that glutamine supplementation should be con-

Table 3 Anti-infective and immunomodulatory properties of immunonutrients
Anti-infective and immunomodulatory properties of immunonutrients
Reduced bacterial overgrowth
Maintenance of natural balance of intestinal flora
Reduced intestinal permeability
Reduced serum endotoxin levels
Antagonist effect against pathogenic bacteria
Prevent pathogenic bacterial adherence to intestinal mucosa
Bacterocidal and bacterostatic effect (lactic acid production)
Increased proportions of NK cells, T-lmphocytes, Ig-A producing plasma cells
Increased phagocytosis

sidered in patients with a critical illness associated with a catabolic response. A meta-analysis published by Asrani *et al*[51], which included 505 patients from 12 studies, demonstrated that glutamine supplementation resulted in a significantly reduced risk of mortality (RR = 0.30; 95%CI: 0.15-0.60; *P* < 0.001) and total infectious complications (RR = 0.58; 95%CI: 0.39-0.87; *P* = 0.009), but not the length of hospital stay (MD = -1.35; 95%CI: -3.25-0.56; *P* = 0.17). However, a clear advantage of glutamine supplementation was seen in patients who received total parenteral nutrition as opposed to enteral nutrition. We drew a similar conclusion when we investigated the effects of intravenous glutamine and early administration of enteral nutrition on SAP outcomes in a prospective randomised controlled trial with 45 patients[52]. This study demonstrated that enteral nutrition supplemented by intravenous glutamine reduced the rate of complications (infected acute and post-necrotic peripancreatic fluid collections, infected pseudocysts and walled-off pancreatic necrosis), but the extent of reduction was not statistically significant. However, the mean hospital stay of the group that received intravenous glutamine and enteral feeding was 10.6 d, significantly shorter than that of the control group, who received enteral feeding alone (15.9 d; *P* = 0.00104).

IMMUNONUTRITION: PROBIOTIC SUPPLEMENTATION

Probiotics are live microorganisms that confer a health benefit to the host, are responsible for the maintenance of the natural balance among gut flora and possess an *in vivo* antagonist effect against pathogenic bacteria. The most widely used probiotic bacteria are *Lactobacillus* and *Bifidobacterium*, which can be isolated from human faeces or intestinal mucosa. Prebiotics are non-digestible food ingredients that are necessary for the propagation of probiotics. Prebiotics selectively stimulate the growth and activity of certain bacteria in the normal gut flora. Synbiotics are nutritional supplements containing both probiotics and prebiotics[53].

Physiologically, some probiotics have been shown to have significant anti-infective and immunomodulatory properties. In addition, they can also prevent pathogenic

bacteria from adhering to the gut mucosa *via* their strong affinity for enterocytes. The complex bacteriostatic and bactericidal effects of probiotics are mainly due to the production of lactic acid and antimicrobial peptides.

Basic data from experimental pancreatitis models initially confirmed the beneficial effects of probiotics. Application of *Lactobacillus plantarum* (*L. plantarum*) reduced the rate of infective necrosis[54], while *Saccharomyces boulardii* with concomitant ciprofloxacin lowered the histopathologic scores of acute necrotizing pancreatitis[55]. Furthermore, probiotics reduced the severity of acute experimental pancreatitis, as well as bacterial translocation to extra-intestinal sites due to a reduction in duodenal bacterial overgrowth, the latter reducing late-phase mortality[56,57].

Similarly, prospective randomised controlled trials have demonstrated beneficial effects of probiotics in acute pancreatitis. Karakan *et al*[58] showed that probiotics reduced the length of hospital stay in enterally fed patients when prebiotics were also applied. Supplementation of enteral nutrients with *L. plantarum* improved clinical outcomes, although control group patients in this study were fed parenterally[17]. In clinical studies, the effects of lactic acid-producing bacteria in acute pancreatitis was investigated for the first time in our department[59]. We found that the rate of pancreatic infectious complications was significantly lower in patients who received live *L. plantarum*. However, mortality was not significantly different between the two groups. We also investigated the use of a combination formula called "Synbiotic 2000" in patients with predicted severe pancreatitis[60]. A decreasing trend in the rate of MOF and septic complications was detected, but these differences did not reach statistical significance. In a multicentre, double-blind, placebo-controlled trial called PROPATRIA organised by the Dutch Acute Pancreatitis Study Group[61], 298 patients with predicted SAP were randomised to receive fibre-enriched enteral nutrition for 28 d with a multispecies probiotic preparation (Ecologic 641) or a placebo. The rate of infectious complications was comparable in both groups, and the mortality rate was higher in the synbiotic group (16% *vs* 6%), which was mainly due to bowel ischemia. Furthermore, organ failure and MOF were more common in the probiotic group (13.2% *vs* 4.9% and 3.0% *vs* 0.7%, respectively), although these differences did not reach statistical significance. Certainly, the synbiotic composition used in the PROPATRIA trial should not be used in critically ill patients[62]. Interestingly, a recent retrospective analysis[63] revealed that probiotic treatment had no apparent negative effect on patients with predicted SAP without initial organ failure, although the authors could not demonstrate the beneficial effects of probiotics in this subgroup of patients. However, the latest randomised controlled trial by Cui *et al*[64] supported the use of probiotics in combination with enteral feeding. The authors compared 70 patients with SAP who received parenteral feeding, enteral feeding or enteral feeding supplemented

with *Bifidobacterium*. They found that the incidence of upper gastrointestinal bleeding, infection and abscess were significantly lower in the probiotic group, and that the length of hospital stay was also significantly shortened in this group. Nevertheless, the results of this study, which was a single-centre study with a relatively low patient number, do not deny the conclusion of the PROPATRIA trial warranting the cautious application of probiotics in SAP. In fact, probiotics cannot be recommended for the management of acute pancreatitis based on the presently available evidence-based data[65,66].

LATEST GUIDELINES

Recently, an International Consensus Guideline was published based on 11 previous guidelines of various societies, which was endorsed by the American Society for Parenteral and Enteral Nutrition[67]. The committee established three categories for the level of evidence in their statements. Importantly, enteral nutrition is generally preferred over parenteral nutrition, and if feasible should be initiated first. Furthermore, continuous enteral nutrition infusion over bolus or cyclic administration is preferred. Similarly, the use of a nasogastric tube for administering enteral nutrition (and the lack of a need to position a postpyloric feeding tube) is also suggested by the committee. Finally, the committee recommends using enteral nutrition in the presence of pancreatic complications such as fistulas, ascites and pseudocysts.

Two other guidelines on the management of acute pancreatitis were published last year, which involved statements on nutritional support during acute pancreatitis. The American College of Gastroenterology guideline recommends enteral nutrition in SAP to prevent infectious complications, whereas parenteral nutrition should be avoided[68]. The most detailed guidelines are based on the collaboration of the International Association of Pancreatology and the American Pancreatic Association involving 121 expert authors, which suggest that enteral nutrition is the preferable method of nutritional support in acute pancreatitis[69]. As far as the composition of nutrients, either elemental or polymeric nutrition formulations can be used. In addition, nasogastric feeding is equally as effective as nasojejunal feeding, and the relevant evidence supports nasogastric feeding. However, a prospective randomised controlled trial called the Study on Nutrition in Acute Pancreatitis is currently underway, which will provide further evidence on nasogastric *vs* nasojejunal feeding (http://clinicaltrials.gov/ct2/show/NCT00580749).

Despite the relatively clear guidelines, everyday clinical practice does not necessarily follow these recommendations. In a recent study by Sun *et al*[70], 43.3% of United States physicians utilised total parenteral nutrition for the treatment of pancreatitis and 36.5% used nasojejunal feeding. Moreover, private physicians use nasojejunal tube feeding in only 19.9% of cases. In a transatlantic survey of nutrition practices in the United Kingdom, the

Republic of Ireland and Canada, 54.2% favoured early feeding in SAP. There was a higher tendency towards enteral nutrition in university hospitals with the nasojejunal route being preferred[71]. In Sweden, enteral feeding is a routine practice in 60% of the hospitals[72]. The positive effects of a national consensus conference were demonstrated by Rebours *et al*[73] in a study involving 176 hospitals in France. While enteral feeding was applied in 25% of the hospitals in 2001, it increased to 58% after the consensus conference.

CONCLUSION

Current evidence confirms that the administration of enteral nutrition is beneficial for the treatment of SAP. Enteral feeding reduces mortality, infectious complications and MOF. As far as the route of enteral feeling is concerned, nasogastric tube feeding is likely to be equally as effective as nasojejunal feeding in SAP. In terms of the timing of enteral nutritional support, relatively early administration within 48 or 72 h of hospital admission is suggested. However, current evidence does not support the application of immunoenhanced nutrients or probiotic supplements, and therefore they cannot be recommended for the management of acute pancreatitis at this time.

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2014 ADVANCES IN PANCREATITIS

Management of chronic pancreatitis complicated with a bleeding pseudoaneurysm

Kun-Chun Chiang, Tsung-Hsing Chen, Jun-Te Hsu

Kun-Chun Chiang, Department of Surgery, Chang Gung Memorial Hospital at Keelung, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Tsung-Hsing Chen, Department of Gastroenterology, Chang Gung Memorial Hospital at Linkou, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Jun-Te Hsu, Department of Surgery, Chang Gung Memorial Hospital at Linkou, Chang Gung University College of Medicine, Taoyuan 333, Taiwan

Author contributions: Chiang KC and Hsu JT designed and performed research; Chiang KC and Hsu JT wrote the manuscript; all authors revised and approved the final edition of manuscript.

Correspondence to: Jun-Te Hsu, MD, Department of Surgery, Chang Gung Memorial Hospital at Linkou, Chang Gung University College of Medicine, No. 5, Fu-Hsing Street, Kwei-Shan Shiang, Taoyuan 333,
Taiwan. hsujt2813@adm.cgmh.org.tw
Telephone: +886-3-3281200-3219 Fax: +886-3-3285818
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Abstract

Chronic pancreatitis is an ongoing disease characterized by persistent inflammation of pancreatic tissues. With disease progression, patients with chronic pancreatitis may develop troublesome complications in addition to exocrine and endocrine pancreatic functional loss. Among them, a pseudoaneurysm, mainly induced by digestive enzyme erosion of vessels in proximity to the pancreas, is a rare and life-threatening complication if bleeding of the pseudoaneurysm occurs. At present, no prospective randomized trials have investigated the therapeutic strategy for this rare but critical situation. The role of arterial embolization, the timing of surgical intervention and even surgical procedures are still controversial. In this review, we suggest that dynamic abdominal computed tomography and angiography should be performed first to localize the bleeders and to evaluate the associated complications such as pseudocyst formation, followed by arterial embolization to stop the bleeding and to achieve early stabilization of the patient's condition. With advances and improvements in endoscopic devices and techniques, therapeutic endoscopy for pancreatic pseudocysts is technically feasible, safe and effective. Surgical intervention is recommended for a bleeding pseudoaneurysm in patients with chronic pancreatitis who are in an unstable condition, for those in whom arterial embolization of the bleeding pseudoaneurysm fails, and when endoscopic management of the pseudocyst is unsuccessful. If a bleeding pseudoaneurysm is located over the tail of the pancreas, resection is a preferential procedure, whereas if the lesion is situated over the head or body of the pancreas, relatively conservative surgical procedures are recommended.

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Key words: Chronic pancreatitis; Pseudocyst; Pseudoaneurysm bleeding; Arterial embolization; Endoscopy; Surgery

Core tip: Chronic pancreatitis complicated with a bleeding pseudoaneurysm is a life-threatening condition. Therapeutic strategies for this rare disease remain controversial. In this review, surgical treatment as a first-line therapy is associated with a high mortality rate in emergency situations. Dynamic abdominal computed tomography and angiography should be performed as the initial management strategy to localize the bleeder, followed by embolization to control the bleeding to achieve early stabilization of the patient's condition. Surgical intervention should be performed for patients who are unable to undergo or who fail arterial embolization for pseudoaneurysm bleeding, or when endoscopic management of the pseudocyst is unsuccessful.

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INTRODUCTION

Chronic pancreatitis (CP) is a long-standing disease characterized by chronic persistent inflammation, leading to permanent duct deformity and insufficiency of both endocrine and exocrine functions. The real incidence and prevalence of CP is hard to estimate due to the lack of exact diagnostic criteria. In general, CP patients present with the classic triad of pancreatic calcifications, steatorrhea, and diabetes mellitus, which are usually seen in very advanced disease. The diagnosis of CP mainly depends on the presentation of typical clinical presentations, imaging findings, pathological features, or exocrine functional insufficiency alone or in combination[1].

The approximate annual incidence of CP is around 7-10 per 100000 people[2]. The causes of CP are complex and involve both environmental and genetic factors. Alcohol consumption, nicotine usage, pancreatic duct obstruction, hyperlipidemia, hypercalcemia, and autoimmune diseases are thought to be the most common causes[1,3,4]. Among them, alcohol has been deemed as the leading cause of CP, accounting for 60% and 35% of CP patients in Western countries and China, respectively[3]. The cumulative incidence of CP was 13% in 10 years and 16 % in 20 years[5]. Whether acute pancreatitis (AP) may progress to CP is still controversial. Postmortem examinations in 405 patients who died of AP showed that the majority of patients had no histological signs of CP[6]. Different experimental models of AP did not provide evidence of AP progressing to CP[7,8]. A study recruiting 532 patients with initial attack of AP with an average follow-up time of 7.8 years showed that the progression from AP to CP occurred only in alcoholics. In addition, smoking significantly enhanced the risk of progression from alcoholic AP to CP[5]. Besides the insufficiency of endocrine and exocrine functions, abdominal pain is the major symptom of CP. Moreover, during CP development and progression, complications such as pseudocyst formation, mechanical obstruction of the gastrointestinal tract and common bile duct, pancreatic ascites, pleural effusion, splenic vein thrombosis with portal hypertension and subsequent varices bleeding, and pseudoaneurysm formation may occur[4]. Due to the recent improvements in endoscopy techniques and instrumentation, most of the abovementioned complications can be treated by endoscopy and medical treatment[9]. The exception is a pseudoaneurysm, which can be a lethal complication of CP if bleeding occurs. For patients without appropriate treatment, the mortality rate may be as high as 90%. Even with a rapid diagnosis and immediate therapy, the reported mortality rate still ranges from 15% to 50%[10,11]. With advances in radiological techniques and instrumentation in the past decade, angiography followed by embolization has been widely used and plays an important role in the diagnosis and management of this situation. To date, therapeutic strategies for CP complicated with pseudoaneurysm bleeding remain controversial. The urgent condition of CP-associated pseudoaneurysm bleeding complicates the management, and thus makes prospective randomized trials difficult. Reports on CP complicated with pseudoaneurysm bleeding are mainly retrospective, and most are from a single institute or limited case reports. Arterial embolization or surgical intervention is currently widely utilized in managing this rare life-threatening condition. In this review, we focus on CP complicated with pseudoaneurysm bleeding with the aim of providing better and reasonable therapeutic strategies to treat this rare but critical situation.

ASSOCIATION OF CP AND PSEUDOANEURYSMS

Pseudoaneurysm formation associated with CP is a rare complication resulting from the erosion of pancreatic or nearby vessels by leaked pancreatic juice. This persistent vessel erosion leads to permanent communication of invaded vessels to the CP-induced pseudocyst, giving rise to the formation of a pancreatic pseudoaneurysm. The majority of related studies have included cases of CP-related vessel injury, which is not associated with pseudocyst formation, as pancreatic pseudoaneurysms for analysis. Three possible mechanisms have been reported to account for the formation of pancreatic pseudoaneurysms: (1) vessel disruption with pseudoaneurysm formation due to severe inflammation and/or pancreatic enzyme autodigestion of a pancreatic or peripancreatic artery; (2) communication of an established pseudocyst and a peripancreatic vessel converting the pseudocyst into a large pseudoaneurysm; and (3) a pseudocyst eroding the bowel wall with bleeding[12,13]. Although the occurrence of a pseudoaneurysm in CP is uncommon, once ruptured it may bleed into the gastrointestinal tract or pancreatic duct, both mimicking gastrointestinal tract bleeding and delaying the correct diagnosis, or directly bleed into the peritoneal or retroperitoneal cavity leading to unstable hemodynamic status and a high mortality rate. The reported incidence of CP complicated with pseudoaneurysm bleeding ranges from 4% to 10%[14-17]. The splenic artery is most frequently involved, followed by the gastroduodenal, pancreaticoduodenal, and hepatic arteries[18].

DIAGNOSIS OF CP COMPLICATED WITH PSEUDOANEURYSM BLEEDING

Since gastrointestinal tract bleeding in patients with CP is usually attributable to stress-related peptic ulcers, erosive gastritis, or varices over the gastric fundus, and as a

pseudoaneurysm may also bleed into the digestive tract, it is difficult to differentiate pseudoaneurysm bleeding from other causes of gastrointestinal tract bleeding in patients with CP. However, the early detection and localization of a bleeding pseudoaneurysm is very important for further management, and key to improving patient survival. Ultrasonography is of little value in this respect, although several studies have demonstrated a successful diagnosis of a pseudoaneurysm using this method[19]. Marshall *et al*[20] reported that dynamic-contrast abdominal computed tomography (CT) should be the first choice of diagnostic tool for CP complicated with a pseudoaneurysm, as it can delineate the anatomy and location of the bleeding pseudoaneurysm in detail. In addition, Balthazar *et al*[21] also suggested that dynamic CT can not only identify the bleeding pseudoaneurysm but also other CP-associated complications. Several studies have shown that angiography is extremely valuable, with sensitivity rates of more than 90% in localizing bleeding pseudoaneurysms[10,18,22,23]. In agreement with their findings, our previous study demonstrated that sonography and dynamic abdominal CT identified pseudoaneurysms in 2 of 5 patients (40.0%) and 4 of 7 patients (57.1%), respectively[24]. Furthermore, angiography led to a correct diagnosis in 7 patients with a sensitivity rate of 100%[24]. Collectively, angiography seems to be a preferential diagnostic tool for CP complicated with pseudoaneurysm bleeding, and we suggest that it should be performed as soon as possible if there is a high index of suspicion of this diagnosis.

ARTERIAL EMBOLIZATION IN CP COMPLICATED WITH PSEUDOANEURYSM BLEEDING

With the enormous improvements in radiological techniques and instrumentation, angiography has been widely used to detect visceral arterial bleeding sites with subsequent arterial embolization to stop the bleeding in the past decade. A radiological vascular approach has been shown to be effective for CP patients with pseudoaneurysm bleeding. In the study of Udd *et al*[17], 33 patients with CP complicated with pseudoaneurysm bleeding were identified from 745 CP patients, and angioembolization successfully stopped the bleeding in 22 of these patients. Only 3 patients experienced rebleeding requiring re-embolization. The success rate varied according to the bleeding site, with an 80% success rate for bleeders around the pancreatic head and 50% for those of the splenic artery[17]. Tulsyan *et al*[25] reported that endovascular treatment of visceral artery aneurysms and pseudoaneurysms was technically successful in 98% of 48 procedures, with a 30-d mortality rate of 8.3% (*n* = 4). However, 3 patients required secondary interventions for persistent flow (*n* = 1) and rebleeding from the previously embolized aneurysms (*n* = 2)[25]. In addition, Bergert *et al*[22] enrolled 35 patients (8 with necrotizing

pancreatitis and 27 with CP) with bleeding pseudoaneurysms treated over a period of 10.5 years with a median follow-up of 4.6 years. Angiographic embolization was performed as the initial treatment in 16 patients (61% embolization rate) with 2 rebleeding, and a mortality rate of 19% for the patients undergoing embolization. In our previous study, 2 of 9 patients with CP complicated with pseudoaneurysm bleeding received emergency angioembolization as first-line therapy[24]. The bleeding was stopped after the procedure, however, both patients developed rebleeding later[24]. To date, the rebleeding rate of pseudoaneurysm bleeding in patients with CP after angioembolization is unclear due to the high heterogeneity of patients recruited in each study. Nevertheless, the application of angiography and angioembolization as first-line therapy to locate the bleeding site and stop the bleeding to stabilize vital signs in patients with bleeding pseudoaneurysms seems to be a reasonable therapy strategy, and is widely used in current clinical practice[17,22,23,26,27].

SURGERY FOR PSEUDOANEURYSM BLEEDING IN PATIENTS WITH CP

Since CP is a disease characterized by ongoing inflammatory processes, some studies recommend that embolization should be considered a temporary procedure to control bleeding, and that subsequent surgical intervention should be conducted as soon as possible to prevent rebleeding[26]. In general, surgical procedures including direct arterial ligation with drainage of a pseudocyst or partial pancreatectomy are used to treat pseudoaneurysm bleeding in patients with CP. Bresler *et al*[12] reported the results of surgical therapy for 10 patients with CP complicated with pseudoaneurysm bleeding. The surgical procedures included transcystic arterial ligation and external pancreatic pseudocyst drainage in 5 patients, distal pancreatectomy in 3 patients, and pancreaticoduodenectomy in 2 patients. One patient (10%) died of sepsis after pancreaticoduodenectomy. However, there was no rebleeding after surgery in their study leading them to conclude that surgical therapy is an acceptable procedure to treat pseudoaneurysm bleeding in patients with CP. Distal pancreatectomy was also recommended for patients with bleeders situated in the tail of the pancreas, while transcystic arterial ligation was suitable for the patients with the bleeder located at the head and body of the pancreas[10]. In another study enrolling 6 patients with CP complicated with pseudoaneurysm bleeding, the authors suggested that primary resection of the pseudoaneurysm should be the treatment of choice[26]. The authors considered that although angiography followed by transcatheter embolization is effective in this regard, it should be considered as a bridging therapy and definite surgical intervention should be conducted as soon as possible, since CP is an ongoing process[26]. Udd *et al*[17] also suggested that patients with unsuccessful arterial embolization should undergo emergency hemostatic

Table 1 Summary of studies concerning chronic pancreatitis-related bleeding

Ref.	Study design	*n*	Intervention	Comments
Gambiez *et al*[10]	Retrospective	14	Angioembolization or surgery	The immediate effectiveness of arterial embolization was remarkable. Subsequent surgery should be reserved for patients in a good general condition with other chronic pancreatitis (CP)-related complications that are not amenable to minimally invasive techniques
El Hamel *et al*[11]	Retrospective	15	Surgery	Favorable results were achieved in two-thirds of patients undergoing primary pancreatic resection which is recommended whenever possible for the treatment of bleeding pancreatic pseudocysts and pseudoaneurysms associated with CP
Udd *et al*[17]	Retrospective	33	Angioembolization or surgery	All hemodynamically stable patients with CP and bleeding pseudoaneurysms should undergo prompt initial angiographic evaluation and embolization if possible. Emergency hemostatic surgery is indicated for unsuccessful embolization
Bergert *et al*[22]	Retrospective	27	Angioembolization or surgery	Angioembolization is effective to stop CP-related bleeding. Partial pancreatectomy is superior to vessel ligation
Hsu *et al*[24]	Retrospective	9	Angioembolization and/or surgery	Angiography is valuable in localizing bleeding pseudoaneurysms. Patients with bleeding pseudoaneurysms associated with CP treated surgically seemingly obtained good outcomes
de Perrot *et al*[26]	Retrospective	6	Angioembolization and/or surgery	Angiography followed by transcatheter embolization is effective to stop bleeding. Pancreatic resection should be performed for the treatment of pseudoaneurysms
Savastano *et al*[27]	Retrospective	8	Angioembolization and/or surgery	Angioembolization is effective to stop acute bleeding in CP to achieve a stable condition quickly. Subsequent surgery is needed to obtain definite treatment
Bhasin *et al*[31]	Retrospective	8	Percutaneous thrombin injection/embolization and endoscopic retrograde transpapillary drainage of pseudocyst	Embolization followed by transpapillary drainage is effective to manage CP patients complicated with a bleeding pseudocyst

surgery with ligation of the bleeding vessel in the head of the pancreas, and distal pancreatectomy for bleeding from the splenic artery or its branch. Furthermore, Bergert *et al*[22] found that ligation or repair of the bleeding vessel was associated with higher rebleeding and reintervention rates, and that partial pancreatectomy was the preferred choice of treatment. In our previous investigation, 7 patients received emergency (*n* = 4) and elective (*n* = 3) surgery as the initial therapy, and all of them survived without rebleeding[24]. In addition, 5 patients underwent surgical treatment for associated pseudocysts, and no cases of rebleeding occurred[24]. With advances and improvements in endoscopic devices and techniques in the last decade, successful endoscopic management of CP-associated pseudocysts has been reported[28-30]. Weckman *et al*[29] reported 170 CP patients with pancreatic pseudocysts who were treated endoscopically, with a success rate of 86% and no procedure-related mortality. Nonetheless, 23 (14%) patients required surgical interventions because therapeutic endoscopy was unsuccessful or technically impossible[29]. In addition, Bhasin *et al*[31] reported 8 CP patients with pseudoaneurysms associated with pseudocysts who underwent radiological arterioembolization or thrombin injections followed by successful endoscopic transpapillary drainage through the major (*n* = 5) or minor papilla (*n* = 3), and resolution of the pseudocysts was noted within 6 wk with no significant complications related to the procedures. Taken together, the role of emergency surgery for bleeding pseudoaneurysms in patients with CP is still controversial. However, considering the substantial surgery-related morbidity

and mortality rates when used as the initial therapy for pseudoaneurysm bleeding in patients with CP under emergency conditions, most recent studies suggest that surgical intervention should be performed in patients who are unable to undergo or who fail arterial embolization for pseudoaneurysm bleeding, or when endoscopic management of the pseudocyst is unsuccessful[17,22,31,32].

CONCLUSION

A pseudoaneurysm is a rare complication in patients with CP, and is caused mainly by erosion of nearby vessels due to digestive enzyme leakage. Life-threatening conditions with subsequent high mortality rates may develop once pseudoaneurysm bleeding occurs without appropriate management. Table 1 summarizes the studies on interventions for CP patients complicated with pseudoaneurysm bleeding and comments on the treatment. To date, therapeutic strategies for pseudoaneurysm bleeding in patients with CP remains challenging and are still under debate due to the lack of prospective randomized trials and the available data having very high heterogeneity with different conclusions. Nonetheless, it is widely accepted that surgical treatment as a first-line therapy is associated with a high mortality rate in emergency situations. Most authors in recent publications suggest that dynamic abdominal CT and angiography should be performed as the initial management strategy to localize the bleeder, followed by embolization to control the bleeding to achieve early stabilization of the patient's condition. Surgical intervention should be per-

formed for patients who are unable to undergo or who fail arterial embolization for pseudoaneurysm bleeding, or when endoscopic management of the pseudocyst is unsuccessful. If a bleeding pseudoaneurysm is located over the tail of the pancreas, resection is the preferred procedure, whereas for lesions situated over the head or body of the pancreas, relatively conservative surgical procedures are recommended.

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2014 ADVANCES IN PANCREATITIS

Study on acute recent stage pancreatitis

Ye-Chen Feng, Min Wang, Feng Zhu, Ren-Yi Qin

Ye-Chen Feng, Min Wang, Feng Zhu, Ren-Yi Qin, Department of Biliary-Pancreatic Surgery, Affiliated Tongji Hospital, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Correspondence to: Ren-Yi Qin, MD, Professor, Department of Biliary-Pancreatic Surgery, Affiliated Tongji Hospital, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jiefang Ave., Wuhan 430030, Hubei Province, China. ryqin@tjh.tjmu.edu.cn
Telephone: + 86-27-83665294   Fax: + 86-27-83665294

Abstract

Acute pancreatitis (AP) is an inflammatory disease of the pancreas which involves the pancreas and surrounding tissue, and systemic inflammation with a characteristic systemic increase of vascular permeability and increased risk of multiple organ dysfunction. Currently, the pathogenesis of AP is fuzzy, and the diagnosis and treatment need to be standardized. Nevertheless, increased knowledge of AP may achieve more thorough understanding of the pathogenesis. The use of further advanced diagnostic tools and superior treatment, potentially will help clinicians to manage AP at an appropriate stage. However, in view of the multi factorial disease and the complex clinical manifestations, the management of patients with AP is also remaining areas for improvement.

Key words: Acute pancreatitis; Organ failure; Necrosis; Inflammation; Management

Core tip: Acute pancreatitis (AP) is a severe disease with high mortality. Increased knowledge of AP may achieve more thorough understanding of the pathogenesis. The use of further advanced diagnostic tools and superior treatment, potentially will help clinicians to manage AP at an appropriate stage.

INTRODUCTION

Acute pancreatitis (AP) annual incidences are reported to range from 5 to 80 cases per 100000 population, which are associated with a startling mortality rate and high annual costs. A number of studies have been conducted every year to elucidate the pathogenesis of AP, to standardize the diagnosis and the treatment of AP. The pathophysiology of AP is complex and involves several inflammatory pathways. The clinical course is usually benign, and clinical signs and symptoms, as well as amylasemia/amylasuria levels, decrease within a few days; however, around 20% of cases develop complications both at the local and systemic levels, with pancreatic necrosis being most common and relevant[1]. At an early stage of the disease an acute inflammatory process of sudden onset occurs in the peripheral and internal areas of the pancreas, which induces multiple organ system dysfunction syndromes in the lung, kidney, liver, and other organs[2]. AP-related mortality still affects around 10% of patients;

half of deaths occur during the first two weeks, usually related to distributive shock and multiple organ failure syndrome; the rest occur later in the course of the disease and result from complications related to the development of pancreatic necrosis and its complications. On account of a better understanding of physiopathology, the improvement of the therapeutic armamentarium, advances in nutritional support[3], dynamic approaches of continuous extra renal replacement techniques, acknowledgement of the central role of pancreatic infection[4], and advances in surgical techniques in improving the inflammatory response in AP[5], AP management has achieved a major breakthrough. However, in view of the multi factorial disease and the complex clinical manifestations, the management of patients with AP is an area for improvement.

PATHOGENESIS

The pathophysiology of AP is complex and involves several inflammatory pathways. The initial trigger is the activation within the pancreatic parenchyma of various proteolytic enzymes, usually promoted by the presence of bile and duodenal contents inside pancreatic ducts[1]. In most western countries 30% to 55% of cases are caused by sludge or gallstones, which are known as biliary pancreatitis[5]. The others are a complication from excess nutrition and alcohol intake. The overproduction of inflammatory mediators (cytokines and non-cytokines) may result in the systemic manifestations of AP[6-8]. Acinar cell damage initiates AP, accounting for local inflammation and local activation of the immune system of the pancreas[6]. Some recent studies have shown that mild AP is associated with extensive apoptotic acinar cell death, whereas acinar cell necrosis with minimal apoptosis is involved in severe AP[9,10].

Pancreatic acinar cells can produce cytokines and chemokines that are involved in the inflammatory response, including the inflammasome-associated factors interleukin-6 (IL-6), IL-18 and caspase-1, which are found in the basolateral region of acinar cells[11,12]. IL-6, which is known to be involved in the signal transducer and activator of transcription 3/suppressor of cytokine signaling-3 (STAT3/SOCS3) cascade, transmits signals by binding to its membrane-bound receptor, IL-6 receptor, and is ubiquitously expressed. The inflammation-associated nuclear factor kappa B induced myeloid cell secreting IL-6, and the effects of IL-6 were mediated by complexation with soluble IL-6 receptor, which is known as trans-signaling. The trans-signaling of IL-6 stimulated phosphorylation of STAT3 and the production of the neutrophil attractant chemokine ligand 1 in pancreatic acinar cells. The expression of cytokines and chemokines, as well as the inflammasome-associated IL-18 and caspase-1, indicate that the inflammatory mediators released during the early response to lipopolysaccharide are produced exclusively by pancreatic acinar cells. In addition, a recent study suggested that the

alcohol-exacerbated lipopolysaccharides response that initiates sub-clinical AP is mediated by acinar cells. Thus, acinar cells are the major source of inflammatory mediators after early pancreatic injury and during the early onset of sub-clinical AP.

Acinar damage by such inflammatory mediators induces the expression of endothelial adhesion molecules and results in a vicious circle that determines an extensive involvement of the vascular endothelium, which in turn generates vasodilation, increased capillary permeability and interstitial edema. In most of these cases the inflammatory process is similar to that of serious sepsis, which leads to multiple organ failure and death. Furthermore, as is the case with sepsis, genetic polymorphisms for some cytokines are associated with prognosis. Meanwhile, free oxygen radicals regulate necrosis extent in acinar cells, the development of pancreatic edema, inflammatory cell sequestration within the pancreas, and the release of inflammation mediators from both acinar and non-acinar cells in the pancreas. The decreased plasma antioxidant levels (total ascorbic acid) and the increased release of lipid peroxidation byproducts are significantly reflected in patients with AP. The body has a number of free oxygen radical-clearing systems, both enzymatic (superoxide dismutase, catalase, myeloperoxidase, and glutathione peroxidase) and non-enzymatic (carotenes, ascorbic acid, tocopherol)[13]. Uric acid, albumin and ascorbic acid represent most of the antioxidant capability of human plasma. The other elements present include bilirubin, a-tocopherol, a-carotene, tryptophan, tyrosine and selenium. The antioxidant is dependent upon the conditions extant in a specific microenvironment at a given time, and the type of oxidative situation[1]. The antioxidant defense system represents a complex network with interactions, synergisms, and specific actions on a given oxidant[14]. A number of studies in animal models have analyzed the association between oxidative metabolism and pancreatic inflammation. Studies in laboratory animals suggest that pancreatic oxidative stress occurs in early stages following induction. Treatment with antioxidant agents has been seen to reduce acinar cell damage and edema in several animal models. This suggests that ongoing free oxygen radical formation reduces antioxidant defensive systems in cells. Regarding the role of bradykinin and nitric oxide, there is controversy in that on the one hand they seem to relieve pancreatic dysfunction by strengthening vascularization and its secretory capacity while on the other there is the notion that nitric oxide may enhance oxidative stress[15]. This mechanism of action in human beings is pending further study.

DIAGNOSIS

The diagnosis of AP requires at least 2 of the 3 features: (1) abdominal pain (epigastric pain often radiating to the left flank and the back); (2) serum amylase and lipase levels at least three times greater than the upper limit of normal; and (3) characteristic findings on contrast-

Table 1 Determinant-based classification of acute pancreatitis severity				
Classification	Mild AP	Moderate AP	Severe AP	Critical AP
(peri) Pancreatic necrosis	No	Sterile	Infected	Infected
Organ failure	No	(and/or) transient	(or) persistent	(and) persistent

AP: Acute pancreatitis.

Table 2 Modified Marshall Scoring System for organ failure					
System	Score				
	0	1	2	3	4
Respiratory (PaO$_2$/FiO$_2$)	> 400	301-400	201-300	101-200	⩽ 101
Renal (serum creatinine, mg/dL)	⩽ 1.4	1.4-1.8	1.9-3.6	3.6-4.9	> 4.9
Cardiovascular (systolic blood pressure mmHg, without inotropic support)	> 90	< 90 Fluid responsive	< 90 Not fluid responsive	< 90 PH < 7.3	< 90 PH < 7.2

PaO$_2$: Partial pressure of oxygen; FiO$_2$: Fraction of inspiration O$_2$.

enhanced computed tomography (CT), magnetic resonance imaging or transabdominal ultrasonography[16]. Sometimes the CT examination is essential to confirm the diagnosis of AP: abdominal pain suggestive for the disease but without serum amylase and lipase levels at least three times greater than normal, which is seen in late presentation of disease in the patient[17]. If AP is on the basis of the first two criteria, contrast enhanced CT may not be necessary in emergency. The onset of AP is defined as the time of onset of abdominal pain and it is not the same as the time of admission to the hospital. The interval between onset of abdominal pain and admission to the hospital should be noted precisely, especially if patients with SAP are transferred to an intensive care unit (second admission) when this type of data are often neglected[16,18,19].

In previous reports, classification of AP was with three subtypes. A web-based institution consultative process revised and updated the Atlanta classification of AP with the involvement of multiple international pancreatic societies[16,19,20]. According to the severity of AP, the disease is classified as mild, moderate, severe and critical by the absence or presence of organ failure and local or systemic complications.

The latest classification of AP: (1) mild AP (MAP) is characterized by the absence of both pancreatic (peri) necrosis and organ failure; (2) moderate AP is characterized by the presence of sterile (peri)pancreatic necrosis and/or transient organ failure; (3) severe AP (SAP) is characterized by the presence of either infected (peri)pancreatic necrosis or persistent organ failure; and (4) critical AP is characterized by the presence of infected (peri) pancreatic necrosis and persistent organ failure (Table 1).

Organ failure is defined for 3 organ systems (respiratory, cardiovascular and renal) based on the worst measurement over a 24-h period. In patients without preexisting organ dysfunction, organ failure is defined as either a score of 2 or more in the assessed organ system using the Sepsis-related Organ Failure Assessment (SOFA) score[21] or when the relevant threshold is breached, as shown: (1) respiratory: partial pressure of oxygen (PaO$_2$) < basal 60 mmHg (with supplementary O$_2$); or PaO$_2$/fraction of inspiration O$_2$ (FiO$_2$) ⩽ 300 mmHg (⩽ 40 kPa); (2) cardiovascular: systolic arterial pressure (SAP) less than 90 mmHg or a reduction of 40 mmHg in basal SAP, with signs of tissue hypoperfusion (lactate > 3 mmol/L); Saturation of central venous oxygen SvcO$_2$ < 70%; and

(3) renal: an increase of basal creatinine by 2 (AKI-2, RIFLE-I) and/or reduction of urinary flow (oliguria) < 0.5 mL/kg per hour × 12 h[22,23].

The most accurate marker in defining the severity of disease is dysfunction/persistent organ failure (lasting over 48 h)[19,24]. The scoring system (in Table 2) was chosen for its simplicity, universal applicability in clinical practice and in research and its ability to stratify disease[19]. Some others like the SOFA scoring system and APACHE II for patients managed in a critical care unit, which includes inotropic and respiratory support, can be used to assess the severity of dysfunction/organ failure. However, for an easier hierarchy, these scores are not included in current classifications[16,19]. A score equal to or greater than 2 in each system defines the presence of organ failure.

The presence or absence of local complications is very important. Local complications of AP are: acute peri-pancreatic fluid collections, acute necrotic collections, pancreatic pseudocyst and walled off necrosis[16,18,19,25]. Other local complications of AP include perturbance of gastric emptying, splenic or portal vein thrombosis, and necrosis of the colon[18,26]. Local complications may be suspected in the presence of recurrent or persistent abdominal pain, increased serum enzymes, worsening of organ dysfunction and/or clinical signs of sepsis (fever or leukocytosis) that require imaging evaluation[27-29].

We think that the accurate description of local complications and of the natural evolution of the disease's specific stages, along with the standardization of terminology will improve the therapeutic management and scientific research data reporting quality.

TREATMENT AND MANAGEMENT OF AP

We recommend the early detection and treatment of AP patients who have organ failure so as to initiate invasive measures to revive the patients as soon as possible. "Potentially severe AP" (PSAP) , which is a new concept in Consensus Statement for intensive care management of AP conference in 2012 was introduced which was defined as a modality of AP which presents one or more organ failures (respiratory problems, renal, arterial hypotension) or alarm signs and it is useful for initial management of

AP. Some of the previously published criteria show that the severity indicated that patients may fail to recover satisfactorily and called these "alarm signs". The "alarm signs" are those forms of symptoms/signs or data in an AP patient that indicate a probable failure to recover well. Alarm signs can be of a radiological, clinical or prognostic scales or analytical nature that were described in the Atlanta classification[23].

The AP alarm signs are the following: (1) clinical: age, obesity, pleural effusion, abdominal defenses, alteration of consciousness; (2) radiological: free peritoneal fluid, pleural effusion; (3) analytical: Hematocrit > 44%, Procalcitonin greater than 0.5 ng/mL during the first 24 h; C-reactive protein (CRP) > 150 mg/L, or a progressive increase in 48 h; and (4) prognosis scales: APACHE-0 > 6; APACHE II > 8; Ranson-Glasgow > 3 points.

Early administration of fluids is recommended in patients with PSAP, mainly during the first 72 h, during which the first 24 h is the most important[30,31]. Regarding the genre of fluid to be administered, colloid *vs* crystalloid, there is no general recommendation for AP treatment, although balanced crystalloid solutions have been observed to control systemic inflammatory response syndrome (SIRS) in PSAP, as well as CRP levels when compared to physiological saline serum[32-34].

The amount of balanced crystalloids should not exceed 4.3 L during the first 24 h treatment. It must be taken with special care when reviving patients with more severe pancreatitis and more comorbidities. Nevertheless, in the first 24 h the administration of more than 3-4 L of fluids seems to be associated with a poor prognosis on account of an increased rate of acute respiratory failure and a greater need for admission to intensive care units, either because of the deleterious direct effects of fluid infusion, or involvement of a patient with complicated AP[35-37]. Stroke volume variation, systolic pulse variation, pulse pressure variation and the overall volume at the end of diastole can be considered useful parameters for assessing IAH patient response to fluid treatment, when taking into account that the response thresholds that distinguish responders from nonresponders can be increased[38,39].

Chronic alcoholic pancreatitis (CAP) and SAP produce a SIRS which result in a highly catabolic, hyperdynamic and hyper-metabolic stress situation[40,41]. The determining factor in patient recovery is previous nutritional status. Implementing total parenteral nutrition and bowel rest has become the classic concept of treating AP. Specialized nutritional support in PSAP, in its CAP and SAP forms, should be utilized early, in the first 48 h after initial resuscitation. If enteral nutrition cannot be administered, due to intolerance to this nutrition, or if it results in an exacerbation of SAP, parenteral nutrition is indicated. SEMICYUC-SENPE (2011) Consensus: a total caloric intake of 25-30 Kcal/kg per day, without exceeding an glucose intake of > 4 g/kg per day, protein intake of 1-1.8 g/kg per day and an intake 0.7-1.5 g/kg per day of lipid. Emerging data suggest that the time, quantity, route and composition of artificial nutrition aim to reduce pancreatic secretion, modulate inflammatory response, prevent and treat malnutrition associated with a severe metabolic-catabolic situation, prevent the development of systemic and local infections in pancreatitis patients[42-45]. In conclusion, nutritional support has become one of the most important factors in the treatment and management of PSAP patients.

Owing to the systemic release of cytokines and pancreatic enzymes, SAP can affect most remote organs by a systemic vascular response. By inhibiting pancreatic secretion, somatostatin and its analogues have been used in severe AP patients, due to their abilities to indirectly reduce the activity of myeloperoxidase[46], reduce release of inflammatory mediators[47], prevent ischemia-reperfusion injury[48] and prevent bacterial translocation[49,50]. Octreotide and its analogues have been recommended in conventional treatment of SAP for a long time, though the actual effects have been discussed[51]. Octreotide treatment is dose-dependent and its effect might be limited by the blood-pancreatic tissue interface, *e.g.*, by ischemia and impaired microcirculation[52-54]. In a recent study, continuous regional artery infusion (CRAI) with octreotide in SAP reduced the pancreatic amylase release into peripheral blood, improved the effects of both local and systemic inflammatory response[55], and confirmed the achievement of octreotide beneficial effects locally in the pancreas.

5-fluorouracil (5-Fu) is considered as an another specific treatment, which has been tried in AP treatment since the 1970s[56,57]. Essentially, 5-Fu can reduce the synthesis of pancreatic enzymes, or serve as a proteinase inhibitor[58]. Continuous regional arterial infusion with 5-Fu can reduce the serum amylase levels in patients. A recent study demonstrated that the combined use of octreotide and 5-Fu, administered *via* CRAI, achieved a synergetic effect in treatment.

The acute necrotizing pancreatitis-induced changes in inflammatory factors and intra-abdominal pressure (IAP) at the intestinal barrier were especially obvious at 6 h post-induction, which is suggested to be an early therapeutic window for AP treatment. The normal value of IAP in noncritical patients is < 0 mmHg and in critical patients is < 12 mmHg. The increase in IAP or in intra-abdominal hypertension (the pressure ≥ 12 mmHg) was detected more than a century ago, which has been known to lead to some alterations in the functioning of the organism[59-62]. The reduction of intra-abdominal pressure is pivotal in preventing AP progress and organ failure, which can be achieved by non-surgical clinical therapies and/or surgical techniques. Non-surgical therapies used to reduce IAP of intestinal contents include prokinetics (erythromycin, neostigmine, metoclopramide), the gastric or rectal probe, relaxation and sedation and the reduction of the third space with diuretics exerted and/or kidney dialysis techniques. If these options fail to reduce and optimize IAP and abdominal perfusion pressure, surgical management should be considered. Among the surgical techniques, percutaneous drainage[63,64] or decompressive laparotomy[65,66] should be considered first in those

cases where there is a quantity of free intraabdominal fluid. If surgical techniques are performed and there is no suspicion of infected necrosis, it is important that no necrosectomy should be performed to prevent it from occurring.

In the 1970s, a group of investigators first proposed a change of strategy in the therapeutic approach of "reducing or mitigating the inflammatory process in the pancreas", by initially using peritoneal lavage[67,68]. With the development of the medical technology, hemofiltration played a critical role in removing inflammatory mediators (IL-1, IL-6, tumor necrosis factor α, platelet-activating factor and complementary fractions) in AP. They obtained consistent data which support the beneficial effects of hemofiltration on the clinical situation and recovery of patients with SIRS or MOF, which conduces especially to the stabilization of the hemodynamic and respiratory systems[69-72]. It is based on some fascinating arguments using hemofiltration as a specific immunomodulating treatment in SAP, such as the positive effect these techniques have on maintaining cellular defense capacity, preventing the development of infections and maintaining the function of certain organs and, finally, improving the possibility of having a positive impact on the prognosis of SAP patients. Early application of continuous veno-venous hemofiltration promotes negative fluid balance and reduction of intra-abdominal hypertension in patients with SAP, without any associated increased infection or mortality rate, and may reduce hospital stay[73].

Nevertheless, there is a consensus on the conservative management of AP patients with sterile necrosis, which is based on traditional medical treatment. Some published studies advocate conservative treatment, even in patients with infected pancreatic necrosis[74-77]. On account of the high mortality rates for patients who are infected, pancreatic necrosis is treated conservatively; this treatment is not advisable unless the patients refuse to adopt pancreatic necrosectomy or are considered inoperable due to some high comorbidities[78]. Radiologically guided percutaneous catheters are applied, which is considered as a "bridge" technique until a more specific treatment can be applied and can obtain a beneficial effect for stabilizing patients who are too serious to tolerate any type of necrosectomy[79]. In a systematic review of the literature on the usefulness of percutaneous drainage as the sole technique in the treatment of patients with pancreatic necrosis, the use of this was found to be adequate for some patients who may not require surgery. A recent national study on patients undergoing surgery shows that necrosectomy for patients with sterile necrosis is associated with increased mortality, meanwhile supporting conservative treatment unless a pancreatic or peri-pancreatic infection is detected[80].

A fraction of patients with non-infected necrosis can benefit from surgical treatment after the acute stage in pancreatitis: (1) after several weeks of conservative treatment, patients who are still suffering from fever, nausea and/or vomiting, lethargy and hyperamylasemia after attempts to return to an oral diet, typically have large amounts of necrotic tissue with concealed retroperitoneal infections that are objectified after debridement[81]; (2) patients who are suffering from postnecrotic rupture of the main pancreatic duct, which is defined as "disconnected duct syndrome", are tributaries of surgical treatment[82]; and (3) organization of necrosis leads to biliary stenosis and/or intestinal obstruction.

Pancreatic necrosis has two distinct phases, the early and late phases, that indicate its dynamic process. The conclusive evidence which advised against necrosectomy of sterile necrosis[83,84] indicates that the best time to perform surgery is during the late phase, often after three or four weeks from the disease onset, in which the necrosis infection is common[79].

Minimally invasive pancreatic necrosectomy[85], which is similar to open necrosectomy, has been developed in surgical treatment, but remains controversial. One group of surgeons support the management of AP, which relegates open necrosectomy to a secondary option after the failure of a minimally invasive technique. On the other hand, there is another group of surgeons who suggest it as a complementary method of open necrosectomy, which is only helpful for the management of waste collection after conventional surgical treatment. Currently endoscopic techniques, with some exceptions, have failed to demonstrate their superiority over conventional techniques; however, the future of minimally invasive techniques in the treatment of patients with infected pancreatic necrosis is promising, as long as the experience in handling it increases and the new technology needed for obtaining the best results appears[82,86].

Due to the physiological stress to patients with SAP with infected necrosis, a laparotomy and open necrosectomy produces a further exacerbation called the "second hit", which brings an increasing mortality rate. Carter's group in Glasgow[87] described retroperitoneal approach which is a classic lumbotomy adaptation for debridement of infected necrosis basically localized on the (peri)pancreas. Some surgeons suggest that compared with open necrosectomy, the operation using retroperitoneal access to the pancreatic area is a minimal access approach for drainage and debridement of infected pancreatic necrosis. Some studies report that this technique does not increase the mortality rate of patients after surgery, which can be applied as many times as necessary, and has advantages over other access approaches.

In summary, patients with sterile necrosis may perform conservational treatment and necrosectomy can be applied in the best time, and who with infected necrosis should be treated surgically based on the clinical situation. Retroperitoneal approach pancreatitis necrosectomy can be performed to reduce "second hit" compared with open surgeries and mortality rate.

CONCLUSION

AP is a disease of high mortality, and thousands of studies about it have been reported in the world. Increased knowledge of the AP may achieve more thorough un-

derstanding of the pathogenesis. The use of further advanced diagnostic tools and superior treatment, potentially will help clinicians to manage AP at an appropriate stage. However, in view of the multi factorial disease and the complex clinical manifestations, the management of patients with AP is an area for improvement.

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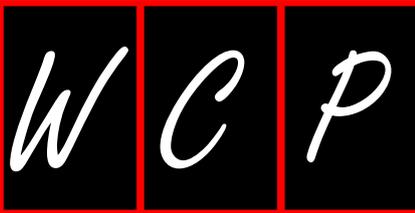
Calcium signaling of pancreatic acinar cells in the pathogenesis of pancreatitis

Jun Li, Rui Zhou, Jian Zhang, Zong-Fang Li

Jun Li, National Local Joint Engineering Research Center of Biodiagnostics and Biotherapy, the Second Affiliated Hospital of Xi'an Jiaotong University College of Medicine, Xi'an 710004, Shaanxi Province, China

Rui Zhou, Jian Zhang, Zong-Fang Li, Department of General Surgery, National Local Joint Engineering Research Center of Biodiagnostics and Biotherapy, the Second Affiliated Hospital of Xi'an Jiaotong University College of Medicine, Xi'an 710004, Shaanxi Province, China

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Correspondence to: Zong-Fang Li, MD, PhD, Department of General Surgery, National Local Joint Engineering Research Center of Biodiagnostics and Biotherapy, The Second Affiliated Hospital of Xi'an Jiaotong University College of Medicine, No. 157 West 5th Road, Xi'an 710004, Shaanxi Province, China. lzf2568@gmail.com

Telephone: +86-29-87678006 Fax: +86-29-87678634

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Abstract

Pancreatitis is an increasingly common and sometimes severe disease that lacks a specific therapy. The pathogenesis of pancreatitis is still not well understood. Calcium (Ca^{2+}) is a versatile carrier of signals regulating many aspects of cellular activity and plays a central role in controlling digestive enzyme secretion in pancreatic acinar cells. Ca^{2+} overload is a key early event and is crucial in the pathogenesis of many diseases. In pancreatic acinar cells, pathological Ca^{2+} signaling (stimulated by bile, alcohol metabolites and other causes) is a key contributor to the initiation of cell injury due to prolonged and global Ca^{2+} elevation that results in trypsin activation, vacuolization and necrosis, all of which are crucial in the development of pancreatitis. Increased release of Ca^{2+} from stores in the intracellular endoplasmic reticulum and/or increased Ca^{2+} entry through the plasma membrane are causes of such cell damage. Failed mitochondrial adenosine triphosphate (ATP) production reduces re-uptake and extrusion of Ca^{2+} by the sarco/endoplasmic reticulum Ca^{2+}-activated ATPase and plasma membrane Ca^{2+}-ATPase pumps, which contribute to Ca^{2+} overload. Current findings have provided further insight into the roles and mechanisms of abnormal pancreatic acinar Ca^{2+} signals in pancreatitis. The lack of available specific treatments is therefore an objective of ongoing research. Research is currently underway to establish the mechanisms and interactions of Ca^{2+} signals in the pathogenesis of pancreatitis.

Key words: Pancreatitis; Calcium signaling; Pancreatic acinar cells; Overload; Cell injury

Core tip: Calcium (Ca^{2+}) overload is crucial in the pathogenesis of pancreatitis, which results in trypsin activation, vacuolization and necrosis. Such cell injury results from increased Ca^{2+} released from intracellular endoplasmic reticulum Ca^{2+} stores, increased Ca^{2+} entry through the plasma membrane and Ca^{2+} pump defects. Current findings have provided further insight into the roles and mechanisms of Ca^{2+} overload in pancreatitis. The lack of specific treatments is a stimulus for ongoing research. This review summarizes recent advances in our understanding of Ca^{2+} signaling in the pathogenesis of pancreatitis, and discusses how research has guided our search for potential therapeutic targets.

Original sources: Li J, Zhou R, Zhang J, Li ZF. Calcium signaling

INTRODUCTION

Pancreatitis remains a disease with significant morbidity and lethality, and is typically caused by alcohol abuse or complications arising from biliary disease[1]. The pathogenesis of pancreatitis is multi-factorial and has not yet been clarified[2-5]. In recent years, several pancreatic mechanisms have been proposed, such as trypsinogen activation[6], pancreatic microcirculation malfunction[7], calcium (Ca^{2+}) overload[8-10] and inflammatory pathways[11-13]. Among these various theories, Ca^{2+} overload is receiving increasing attention and is being extensively investigated in the pathogenesis of pancreatitis[14]. Recent advances in our understanding of Ca^{2+} signaling of pancreatic acinar cells in the pathogenesis of pancreatitis are reviewed in this article, including a discussion on how research has guided our search for potential therapeutic targets.

PHYSIOLOGICAL AND PATHOLOGICAL CA^{2+} SIGNALS IN PANCREATIC ACINAR CELLS

As the most universal carrier of biological signals, intracellular Ca^{2+} is involved in the modulation of virtually all cellular functions, from its origin at fertilization to its end in the apoptotic process[15]. Intracellular Ca^{2+} acts both as a first and a second messenger to control cellular functions *via* regulating free-Ca^{2+} concentrations in the cytoplasm, for example, controlling the contraction and relaxation of muscles, and regulating secretion from exocrine glands[16]. Ca^{2+} signals elicited by physiological stimulation are transient and mostly localized in the granule-containing apical pole, whereas sustained global elevation of cytosolic Ca^{2+} concentrations can be fatal[17-19]. The digestive enzymes produced by pancreatic acinar cells are packaged in zymogen granules in the apical pole[20]. Physiological stimulation elicits proenzyme exocytosis exclusively through the apical membrane[21]. Ca^{2+} overload causes inappropriate intracellular trypsin activation, vacuolization and necrosis[20,22-26], which contribute to subsequent cell injury and are often fatal in human acute pancreatitis[27]. Pretreatment with pharmacological Ca^{2+} chelators or blockers was found to prevent premature digestive enzyme activation, vacuolization, skeletal disruption and acinar cell necrosis induced by Ca^{2+} overload[28].

RELEASE OF CA^{2+} FROM THE ENDOPLASMIC RETICULUM

There are two types of G protein-coupled receptors local-

ized on the plasma membrane, namely, acetylcholine (ACh) and cholecystokinin (CCK) receptors[8]. ACh is a secretagogue that activates phospholipase C (PLC) through ACh receptor ligand binding, which in turn cleaves phosphatidylinositol 4,5-bisphosphate into the classic Ca^{2+}-releasing messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol to mobilize Ca^{2+} and activate protein kinase C respectively[29]. The other principal secretagogue in acinar cells is the hormone CCK, which exists in multiple molecular forms, such as CCK8 and CCK58. CCK interacts with its receptor and activates adenosine diphosphate-ribosyl cyclase to produce the novel Ca^{2+}-releasing agent nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic adenosine diphosphate-ribose (cADPR).

There are two types of regulated Ca^{2+}-release channels localized on the endoplasmic reticulum (ER) membrane, namely, the IP_3 receptors (IP_3R) and ryanodine receptors (RyR). IP_3R are concentrated in the apical part of the acinar cell and binding of IP_3 activates gated Ca^{2+} channels to release intracellular stored Ca^{2+} from the ER, which participates in the apical cytosolic Ca^{2+}-spiking response to stimulation with physiological concentrations of ACh[10,19,30,31]. RyR in the basal region of acinar cells are activated by NAADP and cADPR, and oligomers form gated Ca^{2+} channels to release intracellular Ca^{2+} from ER stores[32] in response to stimulation with physiological concentrations of CCK[33-35]. Intriguingly, the Ca^{2+} response mediated by RyR was observed in the apical pole in mouse acinar cells and required functional IP_3R, which could be interpreted as co-localization and coordination of RyR and IP_3R[36].

Hyperstimulation with agents (in contrast to physiological stimulation) can induce acinar cell injury by IP_3R-induced release of Ca^{2+} from the ER. The Ca^{2+} increase spreads from the apical pole to the basolateral part of the acinar cell, and a sustained global Ca^{2+} elevation causes pancreatitis-like cellular changes, such as abnormal intracellular enzyme activation, vacuolization and necrosis[20]. Treatment with IP_3R inhibitors, such as caffeine and 2-aminoethoxydiphenyl borate, can reduce abnormal Ca^{2+} signals and the probability of ethanol-induced pancreatitis, but the low affinity and multiple actions restrict its therapeutic potential[37,38]. Hyperstimulation by CCK8 is specifically dependent on functional RyR, and induces toxic pancreatitis-like changes as a result of sustained global elevation of Ca^{2+} released from the ER. These aberrant Ca^{2+} signals and acinar cell injuries can be blocked *in vitro* and *in vivo* by pretreating with RyR inhibitors[8,39]. Hyperstimulation by CCK also activates PLC, which generates IP_3 and elicits Ca^{2+} overload[20].

Although the ER is a large Ca^{2+} store in the basolateral part of pancreatic acinar cells, there are also extensive acidic Ca^{2+} stores present in the apical part, which similarly release Ca^{2+} into the cytoplasm through IP_3, cADPR and NAADP signaling. Hyperstimulation from bile acids and alcohol metabolites can elicit pathological Ca^{2+} release from both the ER and acidic stores[40,41].

STORE-OPERATED CA²⁺ (SOC) INFLUX

Another abnormal Ca²⁺ signal in the pathogenesis of pancreatitis is extracellular Ca²⁺ entry, which is regulated at the plasma membrane of acinar cells by SOC channels[42]. Under physiological conditions, CCK and ACh induce the release of Ca²⁺ from the ER, followed by Ca²⁺ extrusion from the cell, suggesting that SOC entry is required to elevate intracellular Ca²⁺. The molecular mechanism underlying these pancreatic Ca²⁺-entry channels is ill-defined. Current research suggests that Ca²⁺-entry channels belong to the transient receptor potential family, including Ca²⁺ release-activated Ca²⁺ channel protein 1 (Orai1), transient receptor potential channel 1, and stromal interaction molecule (STIM) 1[43,44]. Recent studies indicate that STIM proteins serve as sensors, are concentrated in the ER membrane, and monitor the Ca²⁺ concentration in the ER lumen. When the luminal concentration is reduced in response to secretagogue stimulation, STIM proteins sense the changes, accumulate and translocate to the plasma membrane where they co-localize with and activate Orai1 channels[45-48].

Orai1 channels are localized not only in the apical part of acinar cells, but also in the basal and lateral membranes, which cover about 95% of the pancreatic acinar cell surface[43]. Following ER Ca²⁺ store depletion, Orai1 interacts with STIM and activates SOC channels. The wide distribution of Orai1 channels enables sustained Ca²⁺ entry under physiological conditions, without the need for local Ca²⁺ concentrations, and refilling of the ER Ca²⁺ stores after agonist-elicited depletion[8]. However, Orai1 activity can result in an abnormal sustained global Ca²⁺ elevation following pathological stimulation, such as by high, toxic concentrations of CCK8, alcohol and bile acid, which all elicit intracellular Ca²⁺ overload that is mostly dependent on external Ca²⁺ influx[20,23,25]. Therefore, SOC entry may be crucial for the development of acute pancreatitis. Without external sustained global Ca²⁺ entry, cellular injury does not occur[20,22-25,49]. Removal of external Ca²⁺ or abrogation of elevated Ca²⁺ with a Ca²⁺ chelator can protect acinar cells against abnormal changes, such as trypsinogen activation and vacuolization[20,25,49]. SOC channel blockers might therefore be a possible therapeutic approach for the treatment of acute pancreatitis[7].

CA²⁺ PUMP DEFECTS

Sarco/endoplasmic reticulum Ca²⁺-activated adenosine triphosphate (ATP)ase (SERCA) is an ER Ca²⁺ pump which actively re-uptakes Ca²⁺ into the ER lumen to compensate for resting leakage into the cytosol[8,50]. Under normal physiological conditions, the elevation of intracellular Ca²⁺ can activate the SERCA pump[19,27,51], and Ca²⁺ release elicited by stimulation is followed by Ca²⁺ re-uptake. The rate of uptake decreases as luminal Ca²⁺ concentration increases until the uptake rate equals the resting leak rate[8]. Pathological stimulation by bile acids

or fatty acids can elicit Ca²⁺ overload by inhibiting the SERCA pump and depolarizing the inner mitochondrial membrane, resulting in reduced ATP production, which in turn lessens the ability of the SERCA pump to restore ER Ca²⁺ stores[25]. Prolonged and uncompensated Ca²⁺ overload released from ER stores can cause thapsigargin activation and vacuolization in pancreatic acinar cells, which can be visualized directly[20].

All eukaryotic cells export Ca²⁺ through two pathways, the plasma membrane Ca²⁺-ATPase (PMCA; commonly called the Ca²⁺ pump) and the Na⁺-Ca²⁺ exchanger (NCE), to prevent Ca²⁺ overload and for the maintenance of intracellular Ca²⁺ at the appropriately low level[52,53]. The PMCA has high Ca²⁺ affinity but low transport capacity and is ATP-dependent. Any elevation of cytosolic Ca²⁺ can activate the PMCA to rapidly extrude Ca²⁺ in physiological conditions. Whereas ER-released Ca²⁺ is localized in the apical part and Ca²⁺ entry occurs across the basolateral surface, PMCA Ca²⁺ extrusion is confined to a small apical region only, which restricts the PMCA function as a fine-tuner of cell cytosolic Ca²⁺. Pathological stimulation can depolarize mitochondria and cause a deficiency in ATP production, which inhibits Ca²⁺ extrusion and aggravates the cytosolic Ca²⁺ overload[24,54].

The NCE has a low Ca²⁺ affinity and is a high-capacity transmembrane protein of the plasma membrane involved in Ca²⁺ homeostasis, and is especially important in excitable cells. Because of its high capacity, the NCE can extrude Ca²⁺ at a much higher rate than the PMCA, serving as the fast Ca²⁺ transporting system. For example, activation of the NCE prevents Ca²⁺ overload induced by pathological stimulation and cell death in neurons. Inactivation of the NCE can cause neuronal death, which can be visualized directly[55]. In pancreatic acinar cells, the NCE is of little quantitative importance, which explains why Ca²⁺ overloading is particularly dangerous in pancreatic acinar cells[19,27].

As another Ca²⁺ store, mitochondria also participate in maintaining cytosolic Ca²⁺ homeostasis in pancreatic acinar cells. Mitochondria surround the apical pole in a perigranular belt, separating zymogen granules from the basolateral part of the acinar cell, and are also positioned just beneath the plasma membrane and surrounding the nucleus[19,27,55-58]. The membrane potential across the inner mitochondrial membrane is the driving force behind mitochondrial uptake of Ca²⁺ into the matrix through the Ca²⁺ uniporter, a Ca²⁺-selective ion channel[59,60]. Mitochondria in pancreatic acinar cells play an important role in maintaining cytosolic Ca²⁺ homeostasis[56-58]. When cytosolic Ca²⁺ is elevated by physiological stimulation, mitochondria sense the Ca²⁺ in the environment and take up Ca²⁺ *via* the Ca²⁺ uniporter[60]. Ca²⁺ spikes released from the ER occurring in the apical region can cause immediate Ca²⁺ uptake into the mitochondrial matrix, preventing further spread of the Ca²⁺ signal into the basolateral part of the acinar cell, which contains the nucleus. Perigranular mitochondria function as a Ca²⁺ buffer barrier[8], causing Ca²⁺ uptake termination and Ca²⁺
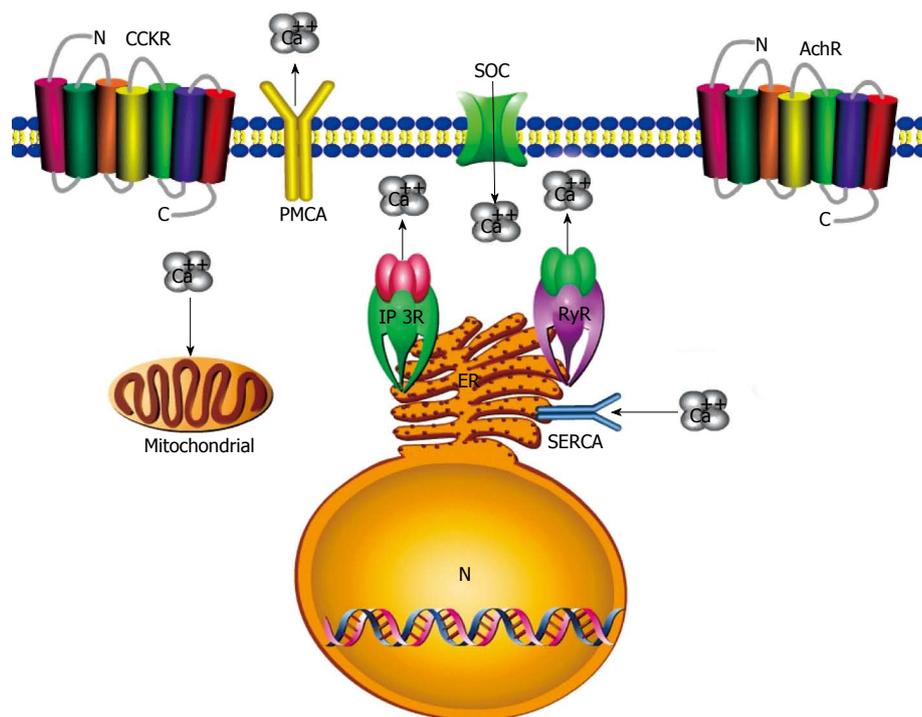
Figure 1 Diagram showing possible interventions for therapeutic targets of pancreatitis. Blockage of Ca^{2+} entry will probably depend on inhibition of the store-operated Ca^{2+} (SOC) channel in the pancreatic acinar cell. Activation of plasma membrane Ca^{2+}-adenosine triphosphate (ATP)ase (PMCA) and sarco/endoplasmic reticulum Ca^{2+}-activated ATPase (SERCA) would enhance Ca^{2+} extrusion from the cell and endoplasmic reticulum (ER). Intracellular Ca^{2+} release from the ER can be prevented through inhibition of the inositol 1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RyR). Preconditioning strategies could protect mitochondrial function to ensure adequate ATP production extrusion by Ca^{2+} pumps and for pancreatic acinar cells to survive intact.

Table 1 Potential therapeutic targets
Potential therapeutic targets
Store-operated Ca^{2+} channel
IP3 receptor
Ryanodine receptor
SERCA
PMCA
Mitochondria

IP3: 1,4,5-trisphosphate; PMCA: Plasma membrane Ca^{2+}-adenosine triphosphate (ATP)ase; SERCA: Sarco/endoplasmic reticulum Ca^{2+}-activated ATPase.

removal *via* the mitochondrial NCE[61,62]. Mitochondrial Ca^{2+} uptake activates Krebs cycle enzymes and drives ATP production, supplying ATP for SERCA-mediated Ca^{2+} re-uptake into the ER and PMCA-mediated Ca^{2+} extrusion[27,63]. Pathological stimulation that can induce experimental pancreatitis, such as with bile salts, fatty acids and CCK or its analog, can depolarize the inner mitochondrial membrane, inducing further collapse of the mitochondrial membrane potential and impairment of ATP production[64]. This situation prevents perigranular mitochondrial Ca^{2+} re-uptake and mitochondria cannot buffer the apical Ca^{2+} elevation, causing the local Ca^{2+} signal to spread to the whole of the acinar cell[30]. Failure of ATP production reduces the ability of the SERCA and PMCA pumps to take Ca^{2+} back into the ER and for extrusion, which contributes to Ca^{2+} overload. This is the most likely explanation for why pretreatment with Ca^{2+} chelators can limit the global and sustained elevation of Ca^{2+}.

TARGETS FOR POTENTIAL THERAPY

To date, there is no specific treatment for either acute or chronic pancreatitis[39,65-67]. The current therapy for pancreatitis is limited to the inhibition of proteolytic enzymes. Protease inhibitors have a modest preventative role in experimental animal models, however, they fail to show therapeutic value in clinical treatment[68,69]. An aberrant increase in cytosolic Ca^{2+} is a key molecular event in the pathogenesis of pancreatitis. Intracellular Ca^{2+} overload is a major reason for pancreatic acinar cell injury from toxin stimulation that induces pancreatitis[7,20,22-25,49]. Abnormal, prolonged, global Ca^{2+} signals lead to premature enzyme activation, vacuole formation and acinar cell damage. Thus, it is clinically relevant to identify the targets of the aberrant Ca^{2+} signals[70]. New avenues are required based on current findings in our understanding of Ca^{2+} signaling in the pathogenesis of pancreatitis (Figure 1). Possible interventions include: (1) inhibition of Ca^{2+} entry pathways; (2) enhancement of Ca^{2+} extrusion; and (3) inhibition of the primary Ca^{2+} release from the ER; and iv) protection of mitochondrial function, which can serve as potential therapeutic targets (Table 1). Recent progress in our understanding of Ca^{2+} signals of pancreatic acinar cells in the pathogenesis of

pancreatitis now provides opportunities for the developments of better therapeutic approaches.

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2014 ADVANCES IN PANCREATITIS

Drug induced acute pancreatitis: Does it exist?

Scott Tenner

Scott Tenner, Division of Gastroenterology, Department of Medicine, State University of New York, Brooklyn, NY 11235, United States

Scott Tenner, Downstate Medical Center, State University of New York, Brooklyn, NY 11235, United States

Author contributions: Tenner S wrote the manuscript based on prior work with Nison Badalov and William Steinberg.

Correspondence to: Scott Tenner, MD, MPH, Division of Gastroenterology, Department of Medicine, State University of New York, 2211 Emmons Ave, Brooklyn, NY 11235, United States. drtenner@brooklyngi.com

Telephone: +1-51-63160830 Fax: +1-718-3682249

Abstract

As the incidence of acute pancreatitis continues to rise, establishing the etiology in order to prevent recurrence is important. Although the etiology of acute pancreatitis is not difficult in the majority of patients, almost a quarter of patients are initially labeled as having idiopathic acute pancreatitis. When confronted with a patient with acute pancreatitis and no clear etiology defined as an absence alcoholism, gallstones (ultrasound and/or MRI), a normal triglyceride level, and absence of tumor, it often appears reasonable to consider a drug as the cause of acute pancreatitis. Over 100 drugs have been implicated by case reports as causing acute pancreatitis. While some of these case reports are well written, many case reports represent poorly written experiences of the clinician simply implicating a drug without a careful evaluation. Over-reliance on case reports while ignoring randomized clinical trials and large pharmaco-epidemiologic surveys has led to confusion about drug induced acute pancreatitis. This review will explain that drug induced acute pancreatitis does occur, but it is rare, and over diagnosis leads to misconceptions about the disease resulting in inappropriate patient care, increased litigation and a failure to address the true entity: idiopathic acute pancreatitis.

Core tip: While the literature has reported over 130 drugs as causing acute pancreatitis, the evidence that these drugs have a true causal role is lacking in the vast majority of drugs. While idiopathic pancreatitis is common, accounting for almost a third of patients with acute pancreatitis, drug induced acute pancreatitis is probably an uncommon, perhaps a rare disease. Before a clinician blames a drug as causing acute pancreatitis, a thorough evaluation for more common causes should be made, even a consideration that the disease is merely idiopathic.

PROBLEM OF IDIOPATHIC PANCREATITIS

Idiopathic Acute Pancreatitis accounts for 20-40 percent of patients with acute pancreatitis[1,2]. That is, normally, approximately a third of patients who present with acute pancreatitis defy the clinician's ability to determine what caused the disease. Idiopathic acute pancreatitis is defined as acute pancreatitis with no etiology established after initial laboratory (including lipid and calcium level) and imaging tests (trans-abdominal ultrasound, MRI and CT in the appropriate patient)[3]. These patients do not have gallstones, a significant history of alcohol use, hyper-triglyceridemia and a tumor. Anatomic and physiologic anomalies of the pancreas occur in 10%-15% of the population, including pancreas divisum and sphincter of Oddi dysfunction[4]. However, it remains controversial if these disorders alone cause acute pancreatitis.

There may be a combination of factors, including anatomic and genetic, that predispose to the development of acute pancreatitis in susceptible individuals[5]. The influence of genetic defects, such as cationic trypsinogen mutation, SPINK, or CFTR mutations, in causing acute pancreatitis is being increasingly recognized. These defects, furthermore, may also increase the risk of acute pancreatitis in patients with anatomic anomalies, such as pancreas divisum[6]. The idea that acute pancreatitis may result from a combination of factors working together should not be a surprise when one considers that most patients with gallstones, hypertriglyceridemia, alcoholism and pancreas divisum will never develop acute pancreatitis.

Clinician's caring for a patient with acute pancreatitis yearn to find a diagnosis to prevent a recurrent attack. This is compounded by the patient's desire to understand what has happened to them to cause so much pain and suffering. In addition to endoscopic interventions, clinicians search the literature for possible causes. The profession demands it, the patient's deserve it, and the literature provides a plethora of possibilities.

DRUGS AS A CAUSE OF ACUTE PANCREATITIS

Most patients who are admitted to a hospital are already taking a medication. Nearly 240 million Americans take at least one prescription drug weekly, and pharmacies fill over ten million prescriptions each day[7]. Over 100 drugs have been reported to cause acute pancreatitis in the scientific literature. Most reviews claim that drug induced acute pancreatitis accounts for 3%-5% of all cases of acute pancreatitis[8]. The diagnosis of drug induced acute pancreatitis is difficult to establish since drug-induced pancreatitis rarely is accompanied by clinical or laboratory evidence of a drug reaction, such as rash, lymphadenopathy, and/or eosinophilia, few ancillary data are available to help with the diagnosis.

While a few medications have been reported to cause acute pancreatitis based on a large body of evidence, most of the drugs implicated are based on case reports that suffer from a combination of inadequate criteria for the diagnosis of acute pancreatitis, failure to rule out more common etiologies, and/or lack of a rechallenge with the medication[9]. A rechallenge is a case in which a patient who develops acute pancreatitis has the medication suspected as causing acute pancreatitis withheld. After the acute pancreatitis resolves, the medication is restarted (typically as the medication was not originally suspected of causing the acute pancreatitis). Within a period of time (typically shorter), after the medication is restarted, the patient has another attack of acute pancreatitis. A valid rechallenge case report should be considered when evaluating whether a particular drug causes acute pancreatitis; however, it is not proof of causation. For example, it is clear that many patients with idiopathic pancreatitis or microlithiasis have recurrent attacks of acute pan-

Table 1 Classification of drug induced pancreatitis
Class I a drugs
At least 1 case report with positive rechallenge, excluding all other causes, such as alcohol, hypertriglyceridemia, gallstones, and other drugs
Class I b drugs
At least 1 case report with positive rechallenge; however, other causes, such as alcohol, hypertriglyceridemia, gallstones, and other drugs were not ruled out
Class II drugs
At least 4 cases in the literature
Consistent latency (75% of cases)
Class III drugs
At least 2 cases in the literature
No consistent latency among cases
No rechallenge
Class IV drugs
Drugs not fitting into the earlier-described classes, single case report published in medical literature, without rechallenge

creatitis. Therefore, stopping and restarting a drug with recurrence of pancreatitis may be a coincidence and not cause and effect[10].

Badalov *et al*[9] published an extensive review of published case reports in the peer reviewed literature. Using criteria based on the presence of a rechallenge, latency, and the number of case reports (Table 1), a classification system "based on the evidence" was provided. Table 2 shows the medications from the published case reports with the "most evidence" of causing acute pancreatitis. At the time the authors published the paper, none of them were aware that the United States Food and Drug Administration ("FDA") and trial lawyers would use the classification as a partial basis for assigning blame to drugs as causing acute pancreatitis[11].

FDA ADVERSE EVENT REPORTING SYSTEM

Through the Federal Food, Drug, and Cosmetic Act ("FDCA"), the FDA is empowered to verify the safety of drugs on the market[12]. Although the FDA employs a rigorous review process to ascertain the safety and efficacy of drugs prior to approval, reports have consistently warned that pre-market research often fails to provide an accurate risk-benefit profile for marketed products[13]. Many drugs come to the market and subsequently are found to have significant side effects that pre-market trials did not reveal[14]. To rectify this problem, the FDA had developed the Adverse Event Reporting System (FAERS)[15].

Based on "MedWatch Reports"[16] filed by interested clinicians, the FDA's reporting programs generate a "deluge of information. Annually the agency has received more than 200000 adverse event reports regarding drugs or biologic products. It is not surprising that the agency describes its analysis of this flood of data as triage[17]. The reports are typically incomplete and often, biased. Although more work on the database and system is need-

Table 2	Summary of drug-induced acute pancreatitis

Class 1a
Azodisalicylate; Bezafibrate; Cannabis; Carbimazole; Codeine;
Cytosine; Arabinoside; Dapsone; Enalapril; Furosemide;
Isoniazid; Mesalamine; Metronidazole; Pentamidine; Pravastatin;
Procainamide; Pyritonol; Simvastatin; Stibogluconate;
Sulfamethoxazole; Sulindac; Tetracycline; Valproic acid
Class 1b
All trans-retinoic acid; Amiodarone; Azathioprine; Clomiphene;
Dexamethasone; Ifosfamide; Lamivudine; Losartan; Lynesterol/
methoxyethynylestradiol; 6-MP; Meglumine; Methimazole;
Nelfinavir; Norethindronate/mestranol; Omeprazole; Premarin;
Sulfamethazole; Trimethoprimsulfamethazole
Class 2
Acetaminophen; Chlorthiazide; Clozapine; DDI; Erythromycin;
Estrogen; L-asparaginase; Pegasparagase; Propofol; Tamoxifen

Table 3	Methods of causal inference

Randomized controlled trials
Controlled trials without randomization
Cohort studies
Case-control studies
Ecologic studies
Case reports and case series

ed to distinguish reliable findings from "variability and noise", more resources are necessary and lacking[18].

Despite incomplete data, the FDA often relying on the FAERS will issue warnings and require manufacturers to add "black box warnings" intended to alert physicians to the importance of the adverse information learned. However, with premature data causing unsubstantiated fears, the FDA has added, modified, and often removed black box warnings from the drugs in question. The addition of these black box warnings has fueled litigation[17].

AN ILLUSTRATION OF THE FALLACY OF DRUG INDUCED ACUTE PANCREATITIS: EXENATIDE (BYETTA®)

The claim that Byetta (exenatide and other GLP-1 agonists) cause acute pancreatitis exemplifies the problem with drug induced acute pancreatitis. Based on case reports, especially following the criteria set forth in the paper by Badalov *et al*[9] MedWatch reports, the FAERS, resultant black box warnings, and poor science, confusion and litigation resulted as "experts" claimed the exenatide caused acute pancreatitis.

Exenatide, an incretin mimetic, was approved as Byetta by the FDA on April 25, 2005. The drug is an adjunctive therapy to improve glycemic control in patients with type II diabetes mellitus. The first published case reports of acute pancreatitis thought to be caused by exenatide appeared shortly after the drug was approved[19]. Additionally, by December 31, 2006, according to the FAERS database, there were 48 documented domestic cases of acute pancreatitis in patients taking exenatide[20]. Noting slightly more cases of acute pancreatitis than expected in the general population, the FDA asked the manufacturer, Lilly and Amylin Pharmaceuticals to strengthen the labeling of acute pancreatitis from the Adverse Reactions section to the Warnings and Precautions section of the exenatide label.

While the FDA was comparing the incidence of acute pancreatitis in the exenatide using diabetic population to the general population, it is not clear that they were aware that diabetic persons were at a significant increased risk of developing acute pancreatitis. For a variety of reasons, including increased incidence of gallstones and hypertriglyceridemia, the incidence of acute pancreatitis in patients with diabetes is higher than the general population[21]. Therefore, regardless of the drug used, if one simply compared the normal population incidence of acute pancreatitis with the diabetic population, one would find a higher incidence in the diabetics. This is a classic confounding variable rather than a drug effect.

The limitations to Medwatch reports cannot be over stated. In many reports the diagnosis of acute pancreatitis is not clearly established. Thus, there is no reason to proceed with considering the case as the adverse event suspected may be another pathology in the abdomen. Misdiagnosis of acute pancreatitis often occurs by clinicians who search for a reason for abdominal pain and merely rely on mild elevations in the amylase and lipase to reach a diagnosis. This is not appropriate, however, as any inflammatory process in the abdomen can cause a mild 2-3 fold elevation of the amylase and/or lipase[22]. Additionally, many patients with diabetes have been shown to have mild elevations, greater than three times the upper limit of normal, of amylase and/or lipase[23]. Thus, many patients with abdominal pain from other sources are falsely labeled as having acute pancreatitis. In the patients who truly have acute pancreatitis, many of the reports fail to identify if the patient has more likely causes of acute pancreatitis, such as gallstones, a history of alcoholism, hypertriglyceridemia[9].

Despite the limitations to the reports and the FDA's position that the FAERS is for hypothesis testing, Elashoff and colleagues[24] examined the FAERS database from 2004-2009 for reported adverse events for exenatide and other medications (which served as controls) in order to determine if patients were at an increased risk of developing pancreatitis. The authors found that the risk of developing pancreatitis from exenatide was higher compared to from other therapies, but importantly the issue of reporting bias could not be entirely ruled out.

Although the FDA agreed to study the issue further, in the meantime it required Amylin and Lilly to alert health care professionals in several ways - including *via* industry letters, published articles, and reports of these cases in the FDA Newsletter[25]. The result was a surge of FAERS cases involving exenatide as a cause of acute pancreatitis immediately followed the FDA notification requirement. Despite the obvious reporting bias induced by the FDA notification, and the failure of the FDA to

Table 4 Bradford Hill criteria for causation

Temporality - causal factor must precede effect

Strength of association - magnitude of the relative risk estimates observed

Consistency of the association - extent to which scientific results are similar across the entire body of evidence

Biologic gradient (dose-response) - the extent to which the relative risk estimates increase in magnitude as the dose of the exposure increases

Biologic plausibility - the extent to which a mechanism of action has been proposed, studied and demonstrated in toxicological or other laboratory based studies

Specificity - refers to the precision with which the exposure and the outcome can be defined

Coherence - the extent to which the evidence and hypotheses for the results fit together into a reasonable and well-tested explanation

Experimentation - the extent to which a randomized clinical trials or cohort studies are available

Analogy - the extent that the purported exposure-disease relationship under consideration is similar to other relationships

note that the population using exenatide-diabetics-inherently had a predisposition for acute pancreatitis, the FDA subsequently added a black box warning to the drug's labeling. The black box warning stated that exenatide could cause acute pancreatitis[26].

Immediately thereafter, thousands of persons who had developed acute pancreatitis while taking exenatide initiated multiparty litigation suits. They relied on the FAERS database and resultant black box warning. The plaintiffs, diabetics already at risk for developing acute pancreatitis, claimed that the defendants Lilly and Amylin Corporations knew or should have known of the hazards associated with exenatide in causing acute pancreatitis. In addition, by claiming that the defendants actively concealed information that demonstrated the dangers of their drug and thus misled the public and prescribing physicians, the plaintiffs were granted broad access to company documents during discovery[27]. The costs of litigation skyrocketed.

Despite the persistent litigation occurring, over the last year, the FDA independently evaluated the post marketing reports that exenatide was a cause of acute pancreatitis. After an exhaustive evaluation of more than 250 toxicology studies conducted in nearly 18000 live animals, no evidence of pancreatic disease was found[28]. In addition to the laboratory data, the FDA reviewed data from 200 trials (including other GLP-1 agonists), involving 41000 patients, and found no evidence of an increased risk of pancreatic disease. The FDA has promulgated that "assertions concerning a causal relationship between incretin drugs are inconsistent with the scientific literature. Simply, despite case reports and MedWatch reports, exenatide does not cause acute pancreatitis.

RETHINKING CAUSATION

It is important to use the general scientific method in making causal claims about human health and disease[29]. The basic structure of the scientific method to determine causation includes: hypothesis generation, observable predictions, alternatives, and tests to distinguish between the causal hypothesis of interest and its alternatives. There could be competing explanations for any scientific observation. Epidemiologic methods involving human subjects are the most important means for identifying and testing hypotheses involving human disease causation. Random-

ized controlled trials are the strongest means and case reports are the lowest means[30] (Table 3). The use of the scientific method avoids falsely claiming causation when the truth is mere chance. Chance is not the only alternative to causation, but must be considered strongly.

The criteria of causation is best understood by the Hill criteria[31]. An "association" in this methodology is not satisfied by the existence of individuals with exposure to the putative cause and the disease of concern. Rather, an "association" from a causal perspective would only exist if a statistically-significant relationship (*e.g.*, between the rate of acute pancreatitis in patients with diabetes mellitus patients exposed to exenatide and the rate of acute pancreatitis in similar diabetic patients not exposed to exenatide) was demonstrated in analytical epidemiological studies. Those studies should be well-designed, with careful attention to diagnostic criteria, adherence to medication, control of confounders, and avoidance (or correction) of important sources of bias. Case reports would never meet this level of evidentiary need to determine causation.

Hill's 9 criteria evaluate the totality of evidence for causation evaluating for temporality, strength of association, consistency of the association, the presence of a biologic gradient (dose-response), biologic plausibility, specificity, coherence, experimentation and analogy (Table 4). In applying the 9 criteria to a drug like exenatide, the evidence shows no causal association. There is no temporality as the latency for exenatide causing acute pancreatitis varies among the reports. As to strength, large epidemiologic studies show no causal relationship of exenatide to acute pancreatitis. There is no consistency of the data. Results from clinical trials, epidemiology, case reports, and animal studies are inconsistent. Based on animal and clinical trial data there is no biologic plausibility (no established mechanism) or gradient. There is no evidence that increase in dosage and/or increase in time results in a linear increase in episodes of acute pancreatitis. Experimental data, in both animals and humans, do not establish that exenatide is a cause of acute pancreatitis. There is also a coherence that exenatide does not cause acute pancreatitis from laboratory, clinical, case report, and epidemiologic studies. Analogy to other anti-diabetic drugs does not strengthen the causal hypothesis as other GLP-1 agonists have also been shown not to cause acute pancreatitis from clinical trials.

Making reliable causal claims in pharmacovigilance is difficult if not impossible when case reports and case series are used as the primary evidentiary source[15]. While the case reports and series generate hypothesis testing, as was shown for exenatide, it is irresponsible to assign causation based on causal hypothesis[32].

DRUG INDUCED ACUTE PANCREATITIS AND IDIOPATHIC ACUTE PANCREATITIS

Although the vast majority of drugs that have been purported to cause acute pancreatitis probably do not, drug induced acute pancreatitis does exist! When evaluating drugs for causation on the basis of the evidence as described by Hill, two drugs meet the evidence of causation: Azathioprine (and its metabolite 6-mercaptopurine) and 2'3'-dideozyinosine (DDI). The strong evidence comes not from case reports but a consideration of the totality of the evidence, including randomized prospective trials, cohort trials, case reports and a molecular basis[33,34]. For example, in the National Cooperative Crohn's Disease study, almost 6% of the 116 patients treated with 6-MP developed acute pancreatitis[35]. Similarly, Haber *et al*[36] treated 400 patients with inflammatory bowel disease with 6-MP and 3.25% developed acute pancreatitis. There are many more randomized trials that support the simplistic case reports.

More recently, Floyd *et al*[34] performed a large population based study including 1388 patients taking azathioprine and 13836 controls in a single county. The incidence rate for acute pancreatitis among all users of azathioprine was one per 659 treatment year. The crude odds ratio (OR) of having redeemed prescriptions for azathioprine within 90 d before admission for acute pancreatitis was 7.5 (95%CI: 2.6-21.6). After adjustment for gallstone disease, alcohol-related diseases, inflammatory bowel disease, and use of glucocorticoids, the OR increased to 8.4 (95%CI: 2.4-29.4). Although there was a significant risk of persons on azathioprine in developing acute pancreatitis, the population-attributable risk, which measures the proportion of all cases of pancreatitis that are attributable to the use of azathioprine in this study population, was 0.4%.

This finding of less than a half percent attributable risk of azathioprine as a cause of acute pancreatitis is extremely important when considering the claims that drug induced acute pancreatitis accounts for 3%-5% of all cases[37-39]. In the absence of data from controlled clinical trials and large pharmacoepidemiologic trials, there is little to no evidence that other drugs cause acute pancreatitis. Although similar data exists for DDI, the drug is not widely used at this time[40,41]. Therefore, drug induced acute pancreatitis probably accounts for less than 1% of cases, and maybe extremely rare in patients who are not taking obvious drugs.

Premarket approval and post-marketing surveillance has become sensitive to determining complications of drugs such as acute pancreatitis. Randomized controlled trials that evaluate for other complications, such as cardiac complications, would detect significant risks of drugs causing acute pancreatitis[42]. In addition, large pharmacoepidemiologic databasis and meta-analyses are often searched for signals to determine whether drugs cause acute pancreatitis[43].

Azathioprine (and 6-MP) and exenatide represent the two extremes of the data demonstrating a causal association for a drug and acute pancreatitis. While there are case reports in the literature and Medwatch reports on the FARS that both drugs cause acute pancreatitis, only for azathioprine (and 6-MP) have multiple randomized controlled trials and large pharmicoepidemiologic studies showing a statistically significant association. For exenatide (and the other GLP-1 agonists), the opposite is true. Multiple controlled trials, pharmacoepidemiologic databases fail to show any causal association with acute pancreatitis.

While clinicians continue to publish case reports blaming drugs as causing acute pancreatitis, it is important to consider the ideas discussed in this paper. Be critical, cynical and remember that idiopathic pancreatitis is common. Clinicians should perform a thorough workup as described to verify the absence of gallstones, alcoholism, hypertriglyceridemia, tumors. However, the struggle to identify a cause, especially in assigning blame to a drug should be done with extreme caution. When a patient asks "what caused my acute pancreatitis?" Clinicians must remember that almost a third of cases will not be clear and are labeled as idiopathic. As clinicians do not have trouble explaining to patients that "bad luck" is the cause of appendicitis, diverticulitis, cholecystitis, telling a patient that it appears simply "idiopathic" may be correct.

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Evidence for a role of mitogen-activated protein kinases in the treatment of experimental acute pancreatitis

Natasha Irrera, Alessandra Bitto, Monica Interdonato, Francesco Squadrito, Domenica Altavilla

Natasha Irrera, Alessandra Bitto, Monica Interdonato, Francesco Squadrito, Department of Clinical and Experimental Medicine, University of Messina, 98125 Messina, Italy

Domenica Altavilla, Department of Paediatric, Gynaecological, Microbiological and Biomedical Sciences, University of Messina, 98125 Messina, Italy

Author contributions: Irrera N and Squadrito F contributed to conception and design; Bitto A, Interdonato M and Altavilla D analysed the data; Irrera N and Bitto A contributed to writing of the paper; Squadrito F, Interdonato M and Altavilla D contributed to revision for intellectual content; Irrera N, Bitto A, Interdonato M, Squadrito F and Altavilla D did the final approval.

Correspondence to: Francesco Squadrito, MD, Professor, Department of Clinical and Experimental Medicine, Section of Pharmacology, University of Messina, Torre Biologica 5th floor, c/o AOU Policlinico G. Martino, Via C. Valeria Gazzi, 98125 Messina, Italy. francesco.squadrito@unime.it

Telephone: +39-90-2213648 Fax: +39-90-2213300

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Abstract

Acute pancreatitis (AP) is an inflammatory disease characterized by acute inflammation and necrosis of the pancreatic parenchyma. AP is often associated with organ failure, sepsis, and high mortality. The pathogenesis of AP is still not well understood. In recent years several papers have highlighted the cellular and molecular events of acute pancreatitis. Pancreatitis is initiated by activation of digestive enzymes within the acinar cells that are involved in autodigestion of the gland, followed by a massive infiltration of neutrophils and macrophages and release of inflammatory mediators, responsible for the local and systemic inflammatory response. The hallmark of AP is parenchymal cell necrosis that represents the cause of the high morbidity and mortality, so that new potential therapeutic approaches are indispensable for the treatment of patients at high risk of complications. However, not all factors that determine the onset and course of the disease have been explained. Aim of this article is to review the role of mitogen-activated protein kinases in pathogenesis of acute pancreatitis.

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Key words: Experimental acute pancreatitis; Mitogen-activated protein kinases; Mitogen-activated protein kinases inhibitors; Cytokines; Cholecystokinin; Cerulein

Core tip: The review focuses on the role of mitogen-activated protein kinases (MAPKs) in the treatment of acute pancreatitis. In fact, acute pancreatitis is a disease characterized by a marked inflammatory reaction and it is usually associated with severe upper abdominal pain, organ failure and also mortality. The activation of MAPKs is an early event in AP and exerts a central role in the onset and development of acute pancreatitis. Thanks to the pivotal function played by MAPKs in acute pancreatitis, the use of specific inhibitors may represent a potential therapeutic target for the treatment of this inflammatory disease.

INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease characterized by acute inflammation and necrosis of the pancreatic parenchyma[1]. AP is often associated with organ failure, sepsis and high mortality. Approximately 20% of patients may develop a more severe form of the disease

with evidence of organ dysfunction[2]. 80% of cases of acute pancreatitis are associated with alcohol excess or gallstones; 10% are idiopathic and a further 10% are related to trauma, biliary interventions and drugs such as antibiotics, diuretics, immunosuppressants and antiretroviral agents. Pancreatitis is associated with parenchymal oedema and apoptosis[3]. The pathogenesis of acute pancreatitis (AP) is still not well understood. In recent years several papers have highlighted the cellular and molecular events of acute pancreatitis. It is now generally known that pancreatitis is initiated by premature activation of digestive enzymes within the acinar cells leading to autodigestion of the gland, followed by a massive infiltration of neutrophils and macrophages and production of inflammatory mediators released from the infiltrated pancreatic connective stroma, such as cytokines, adhesive molecules, platelet activating factors, nitric oxide, oxygen reactive species and lysosomal enzymes that represent the cause of the local and systemic inflammatory response. The hallmark of AP is parenchymal cell necrosis that is responsible for the high morbidity and mortality, so that new potential therapeutic approaches are essential for the treatment of patients at high risk of complications[1,4]. However, not all factors that determine the onset and course of the disease have been explained. Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with several cellular activities as cell proliferation, differentiation, survival, death, and transformation[5]. It has been hypothesized that activation of mitogen-activated protein kinases (MAPKs) is an early event in AP and seems to exert a central role in development and onset of AP[6]. Aim of this article is to review the role of MAPKs in pathogenesis of acute pancreatitis and the potential of MAPKs as therapeutic targets.

MAPKS SIGNALING PATHWAY AND ACUTE PANCREATITIS: THE ROLE OF ERK AND JNK

One of the most important cascades involved in several cellular processes is the mitogen-activated protein kinases (MAPKs) pathway. MAPKs play key roles in signal transduction pathways and are involved in directing cellular response to a variety of stimuli and regulate processes as gene expression, differentiation, mitosis, cell survival, and apoptosis[7-9]. There are three major classes of MAPKs in mammals, the extracellular signal-regulated kinases (ERKs) and the two stress-activated protein kinase (SAPKs) families, c-jun N-terminal kinase (JNK) and p38. MAPKs are activated *via* a signalling cascade that is conserved from yeast to mammals[10,11]. ERK1/2 is mainly activated by mitogens stimuli through the Ras/Raf pathway but can also be activated, independently of Ras, by proinflammatory stimuli including cytokines. JNK and p38 are mainly activated by a variety of stresses and proinflammatory stimuli. Once activated, MAPKs path-

ways orchestrate the recruitment of gene transcription leading to activation of cellular mechanisms such as proliferation, cell differentiation, and inflammation regulated by the release of others growth factors and hormones. In recent years much interest has focused on inhibitors of the mitogen-activated protein kinases (MAPKs) primarily because they have been implicated as key regulators of inflammatory diseases as acute pancreatitis. It has been demonstrated that activation of MAPKs signaling cascades is an early event in AP contributing to the progression of acute pancreatitis[12,13]. Indeed, MAPKs pathways participate to the release of inflammatory mediators highly involved in the development of inflammatory reaction from local to the systemic level[14,15]. At cellular level, time course of MAPKs activation showed that the p38 MAP kinase increases in pancreatic acinar cells most rapidly, with the peak of activity after three hours. JUN kinase activity is the highest after 12 h and after 24 h its activity becomes undetectable[16,17]. Involvement of MAPKs cascade in the pathogenesis of AP is also demonstrated by the fact that hyperstimulation with cholecystokinin (CCK) activates the two isoforms of ERK, p42 and p44, and JNK/SAPK (slowly activated compared with ERK) in pancreatic acini[12,18]. Moreover, CCK activation of JNK/SAPKs results slower than ERK's activation, so that CCK's concentrations for the activation of JNK/SAPKs are higher than the concentrations required for the activation of ERK. Cerulein (CER) is a cholecystokinin-pancreozymin analogue used for experimental acute pancreatitis models in rats and mice, leading to proteolytic enzyme secretion that causes pancreatic acinar autolysis with progressive interstitial oedema just one hour after injection[19]. The stimulation with a low dose of caerulein causes physiological activation both ERKs and JNK/SAPKs. Hyperstimulation both *in vitro* and *in vivo* determines an increase of JNK/SAPKs as a consequence of cellular stress. So, it has been demonstrated that after CCK or CER stimulation *in vitro* as well as *in vivo*, the activation of ERK occurs early than JNK's activation[20]. JNK and ERK1/2 were proposed as important early mediators during caerulein-induced pancreatitis due to their pattern and activation time course[21]. Activation of ERK1/2 and JNK occurs within 5 min, peaks within 30-40 min and decreases, generally, within 1 h following caerulein hyperstimulation. The two MAP kinases cannot be detected anymore 2 h after caerulein injection, thus confirming the very early involvement of this signalling pathway in the inflammatory cascade[22]. Active MAPKs are responsible for the phosphorylation of a variety of effector proteins including several transcription factors that trigger an inflammatory cascade[11,23]. Furthermore, in experimental models it has been shown that also reactive oxygen species (ROS) are responsible for the activation of ERK and JNK in pancreatic acinar cells[24]. In fact, the administration of caerulein *in vivo* stimulates the release of ROS, demonstrating a relationship between increased ROS concentrations and activation of both ERK and JNK[25]. The incubation of pancreatic acini with H_2O_2 causes a dose-

Table 1 Summary of the actions of the mitogen-activated protein kinases inhibitors

Inhibitor	Mechanism of action	Effects
SP600125	Selective and reversible inhibitor of JNK	Dose dependent inhibition of JNK
		Inhibition of inflammatory genes (COX-2, IFN, IL-2, TNF-α) *in vivo*
		Reduction of pancreatic inflammatory mediators (TNF-α, IL-1β) *in vivo*
CEP1347	Potent and selective inhibitor of JNK	Dose dependent inhibition of JNK both *in vivo* than *in vitro*
		Reduction of inflammatory cytokines
PD98059	Inhibitor of ERK 1/2, prevents phosphorylation binding MEK	Protection against inflammatory process in the pancreas *in vivo*
		Protective effects probably related to the inhibition of COX-2
UO126	Selective inhibitor of MEK1 and MEK2; it prevents the activation of ERK1/2	Protection against inflammatory process in the pancreas *in vivo*
SB203580	Selective inhibitor of p38. Inhibition of p38 catalytic activity	Downregulation of the expression of proinflammatory mediators (TNF-α and IL-1β) *in vivo*

JNK: c-jun N-terminal kinase; COX-2: Cyclooxygenase 2; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; ERKs: Extracellular signal-regulated kinases.

dependent, rapid and strong activation of MAPKs: ERK, JNK and p38. These findings underline the potential role of ROS in the pathogenesis of acute pancreatitis, in fact large amounts of ROS are produced near to pancreatic acinar cells[26,27]. Reports describe as ERK can also be activated by exogenous ROS through EGF receptor[28,29]. High concentrations of ROS may cause cytoskeleton disruption in pancreatic acini cells directly and can modify its function *via* activation of MAPKs and p38, so long as these molecules play an important role in the regulation of cytoskeleton function[30]. As described, both inflammatory response and oxidative stress play essential roles on the development of acute pancreatitis, and are correlated with the severity of the disease[31,32]. Pretreatment with an antibody against tumor necrosis factor (TNF)-α or blockade of TNF-α production with pentoxifylline ameliorates experimental AP[33]. The role of oxidative stress in AP has been demonstrated by the beneficial effects of antioxidants[34]. It has been demonstrated that combined treatment by simultaneous blocking of inflammation and oxidative stress pathways has positive effects as therapy in the AP. Blockade of TNF-α production with pentoxifylline partially prevented glutathione depletion and pancreatic inflammation in cerulein-induced AP[35]. Simultaneous inhibition of xanthine oxidase (XO) and TNF-α with oxypurinol and pentoxifylline significantly reduced inflammation in taurocholate-induced pancreatitis[36]. In addition, oxidative stress, as reported, causes activation of MAPKs[37], which activation leads to TNF-α production. In fact, it has been demonstrated that oxypurinol reduces p38 phosphorylation and pentoxifylline reduces ERK and JNK phosphorylation. The combination of the two treatments decreases activation of MAP kinases, and this reduction has been observed in other tissues, such as lung and liver, that are involved in systemic inflammatory process[37]. So, the p38 pathway is related to oxidative stress; ERK and JNK may be associated to inflammatory process and release of pro-inflammatory cytokines. The blockade of these two processes and the concomitant inhibition of MAP kinases can represent a potential therapy to reduce the local and systemic effects in AP, as well as decrease inflammation and production of reactive species

which are involved in development and progression of acute pancreatitis.

PHARMACOLOGICAL MAPKS MODULATION IN AP

Given the role of MAPKs signaling pathway in the development of AP, interest in protein kinases as drug targets has exploded in the past few years, and MAPKs pathways inhibition represents an alternative target in the treatment of AP. Pharmacological inhibitors have been identified which impact on the MAPKs ERK1/2, p38 and JNK/stress activated protein kinases and have been tested in different studies[38], as resumed in Table 1. It has been shown that selective JNK inhibition leads to amelioration of AP. Different JNK inhibitors have been used, among these, SP600125 is one of the most promising inhibitors for treatment of inflammatory diseases involving MAPKs signalling, as acute pancreatitis[39]. SP600125 is a potent, selective and reversible inhibitor of the three JNK enzymes over 300-fold more selective for JNK as compared to ERK1 and p38 MAP kinases, acting through a competitive inhibition with respect to ATP and having an IC50 of 40 nmol/L for JNK1 and JNK2, and 90 nmol/L for JNK3[40]. SP600125 was shown to cause a dose-dependent inhibition of the phosphorylation of c-Jun, and thereby the expression of inflammatory genes cyclooxygenase 2 (COX-2), IFN-γ, interleukin (IL)-2, TNF-α[39]. Minutoli *et al*[41] showed that treatment with SP600125 blunted caerulein-induced pancreatic JNK activation (90%) and partially ERK1/2 activation (45%). The observed greater effect on JNK activity obtained with SP600125 is in agreement with previous "*in vitro*" data showing that this compound exhibits a greater selectivity for JNK as compared to ERK1/2 MAP kinase[39]. In the same study SP600125 reduced the pancreatic content of proinflammatory mediators as TNF-α and adhesion molecules as ICAM-1 with a significant reduction in the oedema and in the inflammatory cell infiltrates, thus confirming the positive effect of MAPKs inhibition on the cell survival during AP[41]. Samuel *et al*[14] provided new evidence that MAP kinases (ERK, JNK, and p38)

are involved in caerulein-stimulated exocrine pancreatic production of cytokines. The group used pancreatic fragments stimulated with caerulein. As awaited, the stimulation wreaked a significant increase of phospho-ERK and phospho-p38. Specific inhibitors of these MAPKs significantly reduced IL-1β and TNF-α production. Using this specific inhibitor of JNK, SP600125, they observed an attenuation of levels of both JNK and IL-1β. Therefore, there is also a connection between the activation of MAPKs and the production of cytokines, responsible for inflammatory events.

Within the MAPKs signaling cascades inhibitors, CEP-1347 is a potent and selective inhibitor of the JNK but not the p38 or the extracellular signal-regulated kinase signalling cascades, studied principally for its neuroprotective effects[42]. The correlation between inhibition of the JNK signaling cascade and pancreatitis amelioration by CEP-1347 is showed in *in vitro* and *in vivo* studies[19,43]. *In vitro* studies demonstrated that CEP-1347 (2 microM) inhibited caerulein-induced JNK activation in a dose dependent manner. Pretreatment of rats with CEP-1347 strongly reduced caerulein-induced pancreatic JNK activation without p38 or ERK inhibition leading to a consequent reduction of pancreatic damage as demonstrated by reduced pancreatic oedema formation and reduced histological severity of pancreatitis. CEP-1347 inhibits JNK activation *in vivo* and ameliorates caerulein-induced pancreatitis. Furthermore, PD98059 and UO126, both inhibitors of ERK1/2, afford significant protection against inflammatory sequelae following experimental acute pancreatitis[44].

Since AP is a condition associated with an inflammatory response, an important role is played by the cytokines TNF-α and IL-1β, which initiate and propagate acute pancreatic inflammation[45]. In fact, patients affected by acute pancreatitis show elevated serum IL-6 levels[46]. IL-6-blocking antibody attenuates experimental pancreatitis and associated pulmonary injury[47].

PD98059 mediates its inhibitory properties by binding to the ERK-specific MAP kinase MEK, therefore preventing phosphorylation of ERK1/2 (p44/p42 MAPK) by MEK1/2, with an IC_{50} values of 4 μmol/L and 50 μmol/L for MEK1 and MEK2. PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf[48]. Similar to PD98059, also U0126 is a selective inhibitor of MAP kinase kinases, MEK1 and MEK2, acting by inhibiting the kinase activity of MEK1/2 thus preventing the activation of MAP kinases p42 and p44. Inhibition of pancreatic ERK1/2 with PD98059 or U0126 *in vivo* protects against the inflammatory sequelae characteristic of the cerulein model of AP[44] confirming the role of ERK1/2 activation in the progression of AP. Moreover, the protective effects of PD98059 might be related to the inhibition of COX-2, although this mechanism has not been well investigated[49].

Evidences have shown that the local pancreatic renin-angiotensin system (RAS) is involved in AP[50]. Angioten-

sin Ⅱ, *via* ROS activation, leads to activation of ERK. Leung *et al*[51] demonstrated in their study the involvement of ERK in regulating angiotensin Ⅱ-induced IL-6 expression in pancreatic acinar cells during pancreatic inflammation. The administration of angiotensin Ⅱ augmented the expression of IL-6, and angiotensin Ⅱ led to ERK activation. The effect of ERK activation has been confirmed using its inhibitor, PD98059. In this model, it has been observed that the activation of ERK is mediated by the release of ROS; in fact, pretreatment with antioxidants reduced ERK activation. Blockade of AT_1 receptors can represent a potential therapeutic approach to the treatment of AP, ROS mediated, too. Using two different inhibitors, SP600125 and PD98059, it has been demonstrated that they completely inhibited the activation of CER-induced pancreatic JNK and ERK[52].

CONTROVERSIAL ROLE OF P38 IN ACUTE PANCREATITIS

Despite the MAPKs have been largely involved in acute pancreatitis, the p38 has an unclear role in the development of the disease. As a matter of fact, studies have suggested that p38 MAP kinase activation could worsen acute pancreatic inflammation or protect against it[43,53]. It has been suggested that the inhibition of p38 exacerbates cerulein-induced pancreatitis in rats[53]. Others experimental evidences demonstrate that the activation, and not the inhibition, of p38 may exacerbate the progression of AP. This kinase regulates activation of nuclear factor (NF)-κB in isolated pancreatic acinar cells, but it is unclear the effective role of p38 MAP kinase in acute pancreatitis.

Moreover, p38 signaling pathway is involved in cytokine-mediated pancreatic beta-cell injury. The activation of p38 MAPK occurs through two different upstream kinases, mitogen-activated protein kinase kinase 3 (MKK3) and MKK6. When activated, it is involved in a lot of responses, such as apoptosis, inflammation and fibrosis[54]. Several studies showed positive effects of systemic p38 inhibitor drugs in a lot of models[55]; other studies demonstrated that systemic p38 blockade could have negative effects[56]. It has been studied the role of MKK3-p38 signaling in a model of cytokine-dependent pancreatic injury induced by multiple low doses of streptozotocin, using mice deficient for the *MKK3* gene[57]. In this study, the group demonstrated that *MKK3* gene deletion has a protective effect, probably due to the suppression of islet inflammation. These findings suggest that MKK3 signaling plays an essential role in the development of pancreatic injury, leading to destruction of beta-cells and hyperglycemia. p38 is activated by CCK in a time and dose dependent manner, with a peak at 5 and 10 minutes, respectively. Twait *et al*[58] expressed a dominant negative form of the p38 MAP kinase (DNp38) and evaluated its effect on NF-κB pathway activation in an exocrine pancreatic cell line (AR42J cells). They observed that DNp38 reduced nuclear translocation of NF-κB and decreased NF-κB-dependent gene transcription after CCK or

TNF-α stimulation in AR42J cells. These results support the hypothesis that p38 regulates transcription factors such as NF-κB in pancreatic exocrine cells[59]. In a recent paper, Wang *et al*[60] investigated the effect of SB203580 which is the inhibitor of p38 mitogen-activated protein kinase on pathologic change of pancreatic tissue and expression of TNF-α and IL-1β in rats with severe acute pancreatitis. This compound is a pyridinyl imidazole inhibitor widely used to elucidate the roles of p38 mitogen-activated protein kinase acting through the blocking of the activation of MAPKAPK-2 by p38 MAPK and subsequent phosphorylation of HSP27[61]. SB203580 inhibits p38 MAPK catalytic activity by binding to the ATP-binding pocket, but does not inhibit phosphorylation of p38 MAPK by upstream kinases. Wang *et al*[60] showed that treatment with SB203580, inhibiting p38 MAPK signaling pathway led to a down regulation of the expression of pro-inflammatory mediators such as TNF-α and IL-1β. All these studies highlighted the central role of MAPKs activation in acute pancreatitis pathogenesis and the real possibility to use pharmacological inhibition of these pathways for treatment of this disease.

OTHER MOLECULAR MECHANISMS INVOLVING MAPKS ACTIVATION IN AP

A number of other molecules participate to the complex network of events triggering the MAPKs activation and the inflammatory response associated with the progression and the onset of AP. In this context, recent advances showed an interaction between p38 and JNK activation and cannabinoid receptor 1 (CB1) and 2 (CB2) in pancreas, where non selective CB1/CB2 agonist HU210 ameliorated experimental pancreatitis[62]. However, the real role of CB1 and CB2 in acute pancreatitis has not been totally investigated. The agonist HU210 carries out a protective effect in pancreatitis also in CB1 deficient mice, and the selective CB2 antagonist, AM630, activates JNK and increases apoptosis in acute pancreatitis. The administration of cerulein in CB1 deficient mice is not responsible for a more severe pancreatitis, if compared to wild type animals, excluding a prominent role of CB1 receptor in the development of the disease[63]. On the other hand the protective effect of CB2 receptor seems to be due to the inhibition of cytokines involved in inflammatory processes, for example, IL-6, which is an activator of JNK[64]. MK2 is a downstream target of p38; the genetic disruption of the *MK2* gene protects against cerulein-induced pancreatitis[65]. Several experiments with MK2 deficient mice have suggested a connection between MK2 and JNK activation: the presence of MK2 determines the activation of CB2 that causes consequently the inhibition of JNK and therefore the attenuation of acute pancreatitis. In MK2 deficient mice, the absence of MK2 creates opposite effects when CB2 receptor is activated, and leads to activation of JNK and increase of IL-6 levels. So, the activation of CB2 receptor has probably protective effects through inhibition of MAPKs cascade in experimental

acute pancreatitis and the use of CB2 agonist can represent an interesting therapeutic target for humans.

In the complex molecular network involved in the regulation of inflammation during AP seems to have a role also protease-activated receptor 2 (PAR2)[66], a member of the G protein coupled receptor superfamily, that plays important roles not only stimulating pro-inflammatory response but also mediates anti-inflammatory effects[67]. PAR2 is activated by activated trypsin in acute pancreatic inflammation; it has pro-inflammatory effects since activates immune and endothelial cells[68]. The protective effects of PAR-2 in acute pancreatitis were investigated in the cerulein-induced pancreatitis model. It has been demonstrated that PAR-2 can activate MAPKs[69]. In contrast, it has been shown that of PAR-2 activation decreases the cerulein-induced activation both ERK and JNK by accelerating their dephosphorylation, activating MAP kinase phosphatases (MKPs), in rat's pancreas. The expression of MKPs provides a negative feedback mechanism for MAP kinases, and the induction of MKP's expression may be activated both by PAR2 and by cerulein. It has been demonstrated that the protective effect obtained by using ERK's and JNK's inhibitors is similar to the effect observed with PAR2 activation, and ameliorates the course of acute pancreatitis[68].

An additional molecule involved in the progression of acute pancreatitis is pancreatitis-associated protein (PAP1). PAP1 is not expressed under physiological conditions whereas is overexpressed during acute pancreatitis[69]. Its activation is linked to a large number of diseases such as inflammatory bowel disease, Alzheimer's disease, and cancer[70-72]. The peak of expression of PAP1 in pancreatic tissue or juice has been observed 24 h after the induction of acute pancreatitis by cerulein[73]. In pancreatic acinar cells the augmented expression of PAP1 led to an increase of resistance to apoptosis[74,75]. Ferrés-Masó *et al*[76] demonstrated an anti-inflammatory role of PAP1, since its induction occurs during inflammatory diseases (pancreatitis, Crohn's disease, ulcerative colitis). *In vivo* studies showed that the administration of anti-PAP1 antibodies worsened the inflammatory response. Treatment with PAP1 prevented TNF-α-induced NF-κB activation in macrophages. Gironella *et al*[70] furthermore demonstrated the anti-inflammatory role of PAP1 in a PAP1-deficient mice model. The anti-inflammatory mechanism of the protein is related to the activation of JAK/STAT3 pathway. PAP1 increases the transactivation activity of the nuclear transcriptional factors associated with MAPKs family. *In vitro* experiments on AR42J pancreatic acinar cell line showed a time-dependent induction of PAP1 gene expression after addition of PAP1 to the colture cells. It has been shown that this cellular line presented basal levels of expression of the proteins members of the MAPKs family: ERK, JNK and p38. Treatment with PAP1 enhanced the phosphorylation of MAP kinases, underlining that PAP1 signal transduction involves MAPKs family[76]. Treatment with MAPK specific inhibitors, such as SB203580 (p38 MAPK inhibitor), PD98059 (ERK inhibitor) and JNK inhibitor,
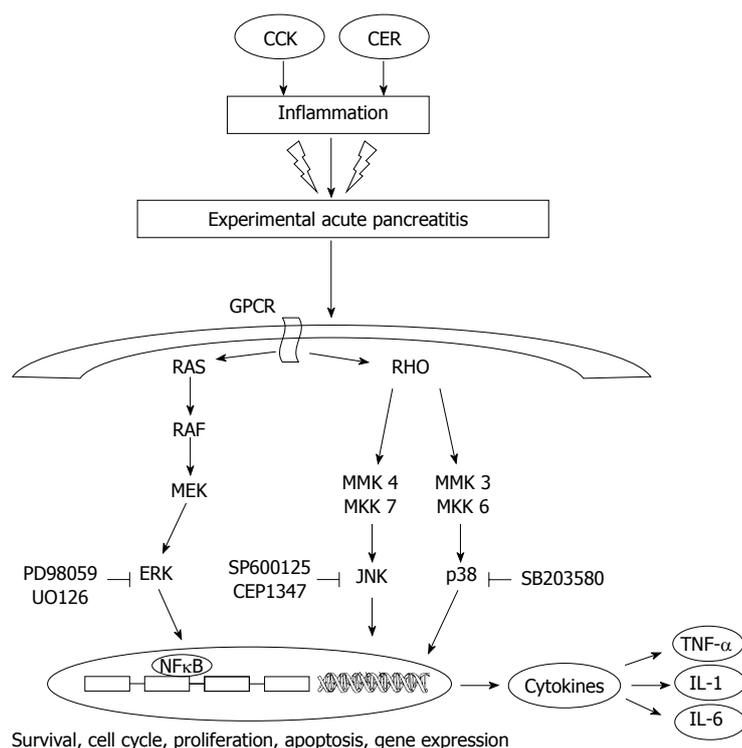
Figure 1 Involvement of mitogen-activated protein kinases and their inhibitors in pancreatic damage. CCK: Cholecystokinin; CER: Cerulein; GPCR: G protein coupled receptor; JNK: c-jun N-terminal kinase; TNF: Tumor necrosis factor; ERKs: Extracellular signal-regulated kinases; NF: Nuclear factor; IL: Interleukin; NF: Nuclear factor.

caused the inhibition of the activation of PAP1. This result demonstrates that the involvement of MAPKs family is essential for the synthesis of PAP1. Some reports indicate that ERK mediates STAT3 phosphorylation both *in vivo* and *in vitro*[77]. Probably a linkage exists between MAPK and JAK/STAT3 pathway upon activation by PAP1.

Also Substance P (SP)[78,79], a neuropeptide released from nerve endings in many tissues, plays an important role in inflammatory processes. SP binds to a G protein-coupled receptor, neurokinin-1 receptor (NK1R). Pancreatic acinar cells express NK1R, SP has been found in pancreas[80], and levels of SP and NK1R are increased in AP[81]. It has been demonstrated that genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury. Knockout mice deficient in the preprotachykinin-A gene, which encodes for SP, are protected against AP[82]. These evidences suggest an important interaction between SP and NK1R in development of acute pancreatitis and lung injury. Studies have shown that SP induces an increase of cytosolic calcium, and probably elevated concentration of calcium is one of the causes of AP[83]. Pancreatic acinar cells treated with SP showed an upregulation of phosphorylation of both ERK and JNK. The inhibitor U73122, a PLC inhibitor, decreased phosphorylation of ERK and JNK, as well as inhibited the activation of NF-κB[84]. These findings are important to demonstrate that drugs targeting SP could represent a therapeutic approach for the treatment of AP.

CONCLUSION

Acute pancreatitis is an autodigestive disease resulting in acute inflammation of the pancreas and MAPKs have been demonstrated to play a pivotal role in the development of the disease (Figure 1). As a consequence of the above reported observations, it is possible to speculate that the blockade of MAPKs may represent a strategic target for future treatment of acute pancreatitis.

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Imaging tests for accurate diagnosis of acute biliary pancreatitis

Valeriu Șurlin, Adrian Săftoiu, Daniela Dumitrescu

Valeriu Șurlin, 1st Clinic of Surgery, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Dolj, Romania
Adrian Săftoiu, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Dolj, Romania
Daniela Dumitrescu, Department of Radiology, University of Medicine and Pharmacy of Craiova, 200349 Craiova, Dolj, Romania
Author contributions: Șurlin V, Săftoiu A and Dumitrescu D contributed equally to the conception and design of the review, aquisition, analysis and interpretation of data; Șurlin V and Dumitrescu D wrote and drafted the manuscript; Săftoiu A revised it critically and gave final approval of the version to be published.
Correspondence to: Adrian Săftoiu, MD, PhD, MSc, FASGE, Professor of Diagnostic and Therapeutic Techniques in Gastroenterology, Research Center of Gastroenterology and Hepatology, University of Medicine and Pharmacy Craiova, 200349 Craiova, Romania. adrian.saftoiu@umfcv.ro
Telephone: +40-251-310287 Fax: +40-251-310287
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Abstract

Gallstones represent the most frequent aetiology of acute pancreatitis in many statistics all over the world, estimated between 40%-60%. Accurate diagnosis of acute biliary pancreatitis (ABP) is of outmost importance because clearance of lithiasis [gallbladder and common bile duct (CBD)] rules out recurrences. Confirmation of biliary lithiasis is done by imaging. The sensitivity of the ultrasonography (US) in the detection of gallstones is over 95% in uncomplicated cases, but in ABP, sensitivity for gallstone detection is lower, being less than 80% due to the ileus and bowel distension. Sensitivity of transabdominal ultrasonography (TUS) for choledocolithiasis varies between 50%-80%, but the specificity is high, reaching 95%. Diameter of the bile duct may be orientative for diagnosis. Endoscopic ultrasonography (EUS) seems to be a more effective tool to diagnose ABP rather than endoscopic retrograde cholangiopancreatography (ERCP), which should be performed only for therapeutic purposes. As the sensitivity and specificity of computerized tomography are lower as compared to state-of-the-art magnetic resonance cholangiopancreatography (MRCP) or EUS, especially for small stones and small diameter of CBD, the later techniques are nowadays preferred for the evaluation of ABP patients. ERCP has the highest accuracy for the diagnosis of choledocholithiasis and is used as a reference standard in many studies, especially after sphincterotomy and balloon extraction of CBD stones. Laparoscopic ultrasonography is a useful tool for the intraoperative diagnosis of choledocholithiasis. Routine exploration of the CBD in cases of patients scheduled for cholecystectomy after an attack of ABP was not proven useful. A significant rate of the so-called idiopathic pancreatitis is actually caused by microlithiasis and/or biliary sludge. In conclusion, the general algorithm for CBD stone detection starts with anamnesis, serum biochemistry and then TUS, followed by EUS or MRCP. In the end, bile duct microscopic analysis may be performed by bile harvested during ERCP in case of recurrent attacks of ABP and these should be followed by laparoscopic cholecystectomy.

Core tip: Gallstones represent the most frequent aetiology of acute pancreatitis estimated between 40%-60%. Clearance of lithiasis (gallbladder and common bile duct, CBD rules out recurrences. Confirmation of biliary lithiasis is done by imaging. Endoscopic ultrasonography (EUS) seems to be a more effective tool to diagnose acute biliary pancreatitis rather than endoscopic

retrograde cholangiopancreatography, which should be performed only for therapeutic purposes. As the sensitivity and specificity of computerized tomography are lower as compared to state-of-the-art magnetic resonance cholangiopancreatography or EUS, especially for small stones and small diameter of CBD, the later techniques are preferred nowadays.

INTRODUCTION

Gallstones represent the most frequent aetiology of acute pancreatitis in many statistics all over the world. The proportion from the total number of acute pancreatitis cases is estimated between 40%-60%, with variations due especially to diagnostic efforts and availability of imaging tests[1]. Accurate diagnosis of acute biliary pancreatitis (ABP) is of outmost importance because clearance of lithiasis (gallbladder and common bile duct, CBD) rules out recurrences, very frequent otherwise, with 30% to 50% of the patients developing recurrent acute pancreatitis relatively soon after discharge (average time 108 d), some of them maybe more severe than the previous episode[2].

Once the diagnosis of acute pancreatitis is made, grounded on generally acknowledged criteria of abdominal pain and three times more than normal hyperamilasemia/hyperlipidemia and/or intravenous (*iv*) contrast-enhanced helical computerized tomography (CT) scan/magnetic resonance imaging (MRI)/ transabdominal ultrasonography (TUS), the biliary aetiology is suspected if jaundice, elevated alanine aminotransferase (ALT) (three times more than normal) or a dilated CBD are present[3]. To those criteria we might add statistical data of a higher incidence in women, between 50 and 70 years of age[1].

Confirmation of biliary lithiasis is done by imaging. Clearance of biliary lithiasis implies a cholecystectomy and the removal of CBD stones. The minimal invasive approach is preferred nowadays, either by combined approach of laparoscopic cholecystectomy and endoscopic extraction of CBD stones, or total laparoscopic approach (cholecystectomy and CBD exploration and calculi extraction). Thus, once a diagnosis of gallbladder lithiasis is made, especially for microlithiasis, the most important thing is to establish whether there is also a CBD stone. Over 90% of the CBD stones come from the gallbladder through the cystic duct. Primary stones arising in the CBD are rarer and usually due to conditions that alter the normal flow of the bile and create conditions for bile stasis. "Silent stones" in the CBD may be present in up to 15% in patients younger than 60 years undergoing cho-

lecystectomy, and even more frequent in older patients[4]. However, the incidence of ABP in choledocholithiasis is only 3%-8%[1]. Even more important, after triggering the acute pancreatitis, most of stones pass through the papilla into the duodenum[5]. Thus, the percentage of CBD stones in ABP decreases from 28.6% in the first 4 h to 8% at 1 wk[6,7].

IMAGING TESTS

Transabdominal US
The first, and the most available and commonly performed is TUS. It seeks for lithiasis in the gallbladder, CBD or indirect signs of biliary obstruction, *e.g.*, dilation of the CBD. The sensitivity of the US in the detection of gallstones is over 95% in uncomplicated cases, but in ABP, sensitivity for gallstone detection is lower, being only 67%-78% due to the ileus and bowel distension[8]. Sensitivity of TUS for choledocholithiasis varies between 50%-80%, but the specificity is high, reaching 95%[9].

Diameter of the bile duct may be orientative for diagnosis. In a prospective study, the diameter of the CBD was measured before cholecystectomy and it was compared afterwards with finding stones at the surgical intervention. There were no stones in the CBD if the diameter was less or equal to 3 mm, while 7.7% of patients with the ducts measuring 4 mm or more had stones. If the size increased, the probability of having stones also increased, nearly all ducts of 9 mm or more had stones[10] (Figure 1).

Endoscopic US
Endoscopic US is more accurate than transcutaneous US, with a sensitivity of over 90% and an even higher specificity[11,12]. Nevertheless, the technique is more expensive and it requires a longer learning curve. EUS seems to be a more effective tool to diagnose ABP rather than ERCP, which should be performed only for therapeutic purposes. In a systematic review of clinical trials from 1994 to 2010, comparing EUS and ERCP in ABP, it was found that EUS avoided ERCP in 71.2% of cases, had no related complication, while ERCP was complicated in over 20% of cases. The clinical course of ABP was not influenced by either of those explorations[13]. A meta-analysis performed on 36 studies with 3532 patients revealed a sensitivity of 89% and a specificity of 94% for choledocholithiasis[14], with another meta-analysis performed on 2673 patients showing even higher numbers of 94% sensitivity and 95% specificity[15]. Consequently, EUS is an important diagnostic tool for the presence of CBD stones, as it accurately visualizes the CBD without the need of instrumentation[16]. There is now enough evidence to support the use of EUS before ERCP, even for smaller stones (less than 4 mm), as it can spare at least two thirds of ERCPs[17]. Moreover, as compared to MRCP, EUS has the same sensitivity, specificity and accuracy, although the sensitivity of MRCP seems to diminish in small (less than 6 mm) CBD stones. Thus,

Figure 1 Large, conglomerated stones into a dilated common bile duct (over 12 mm).
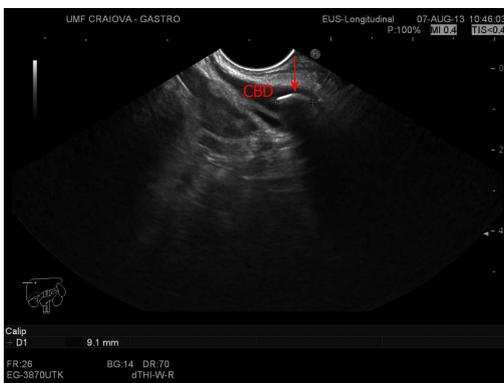
Figure 2 A 9 mm stone, within a slightly dilated, elongated common bile duct.

EUS has a significant impact for surgical decision making, especially in the patients with suspected ABP[18] (Figure 2).

CT

Unenhanced helical CT scan has a variable accuracy for the detection of choledocholithiasis, with a sensitivity of 60%-87% and a specificity of 97%-100%[19,20]. CT-cholangiography has a higher performance for the diagnosis of choledocolithiasis with a sensitivity of 85%-96% and a specificity of 88%-98%[19,21]. As the sensitivity and specificity of CT are lower as compared to state-of-the-art MRCP or EUS, especially for small stones and small diameter of CBD, the later techniques are nowadays preferred for the evaluation of ABP patients.

MRCP

MRCP has a high reported accuracy in the diagnosis of choledocholithiasis. Meta-analyses report pooled sensitivities of 92%-94%[7,22] and a specificity of 99%. There are still controversies regarding the optimal imaging method in the preoperative assessment of patients with ABP, but MRCP has the advantage of a non-invasive method that could properly detect CBD lithiasis. The efficacy of MRCP in detecting CBD stones and to assess the time of choledochal passage of calculi was also compared to
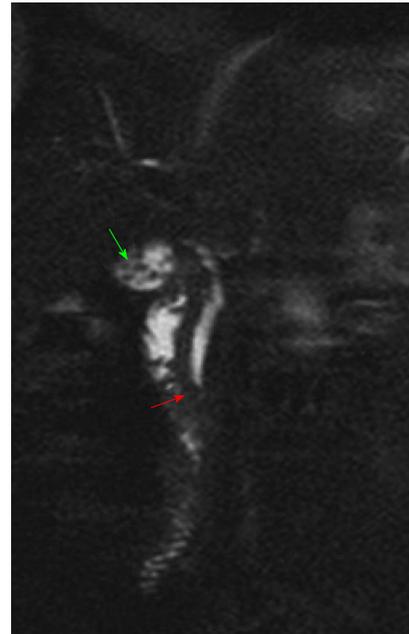
Figure 3 Multiple gallstones in T2 hyposignal less than 5 mm diameter (green arrow), diameter of the common bile duct 6 mm, with a 4 mm migrated stone (red arrow).

ERCP. Overall, MRCP had a positive predictive value 90.5%, negative predictive value 95.2%, sensitivity 82.6%, specificity 97.5% and overall accuracy 94.2%. Moreover, MRCP diagnoses anatomical variants of cystic duct and acute cholecystitis[6,7]. A prospective study compared the efficacy of EUS compared to MRCP and ERCP in the same patients with suspected extrahepatic biliary disease, taking into account also the economic aspect. Results regarding choledocholithiasis were that EUS was more sensitive than MRCP in the detection of choledocholithiasis (80% *vs* 40%), with similar specificity. Rate of acute pancreatitis after ERCP was 6.6%. EUS strategy had the greatest cost-utility by avoiding unnecessary ERCP examinations[23]. Nevertheless, a systematic review showed a similar diagnostic value for prospective studies that compared MRCP and EUS for the detection of CBD stones[24] (Figure 3).

ERCP

ERCP has the highest accuracy for the diagnosis of choledocholithiasis and is used as a reference standard in many studies, especially after sphincterotomy and balloon extraction of CBD stones. Diagnostic ERCP does not, however, detect all stones and in one study its sensitivity was 89% in comparison with EUS, especially for small stones hidden by contrast injection[12]. EUS has been compared to ERCP in a prospective randomized fashion in cases of acute pancreatitis suspected to have a biliary cause. The patients had EUS or ERCP examinations within 24 h from admission. If EUS detected choledocholithiasis, therapeutic ERCP was performed immediately. EUS was successful in all patients, but ERCP failed in 10%, the difference being significant. Also ERCP

failed to identify stones in 8.5%. Morbidity, hospital stays and mortality was similar in both groups[25]. The preferred approach for concomitant gallbladder and CBD stones in the laparoscopic era is sequential preoperative ERCP followed by laparoscopic cholecystectomy, although this has been found to have similar efficacy, maybe with a shorter hospital stay with laparoscopic CBD exploration during cholecystectomy[26]. The same conclusion was also reached by a Cochrane systemic review comparing the endoscopic versus surgical treatment of CBD stones, with laparoscopic CBD clearance being as effective as pre- or post-operative ERCP[27].

Laparoscopic ultrasonography

Laparoscopic ultrasonography (LUS) is a useful tool for the intraoperative diagnosis of choledocolithiasis. Thus, LUS was compared to laparoscopic cholangiography with the same specificity (100%) and positive predictive value (100%), and a sensitivity of 93%[28]. Nevertheless, laparoscopic exploration of the bile duct is as safe and effective as postoperative ERCP in clearing stones from the common duct[29]. The benefit of routine intraoperative cholangiography at the time of cholecystectomy in patients with ABP submitted to laparoscopic cholecystectomy was also questioned. Thus, patients with ABP submitted to cholecystectomy with or without intraoperative cholangiography and CBD exploration were compared in terms of outcome. At 3.8 years of follow up there was no significant difference regarding the rate of recurrent pancreatitis or biliary complications, suggesting that intraoperative cholangiography does not improve outcome after cholecystectomy for gallstone pancreatitis[30]. Another study showed that laparoscopic cholecystectomy (LC) can be performed safely without intraoperative cholangiography (IOC). Thus, from the patients with symptomatic gallstone disease, about 9.2% were selected for preoperative ERCP based upon preoperative clinical, laboratory and ultrasound criteria. In those patients, 58% were found with choledocholithiasis, and stone clearance was achieved in all cases. The other patients were submitted to laparoscopic cholecystectomy with no injury of CBD, no mortality and a rate of retained CBD stones of 1.5% at 2 years follow-up[31].

The necessity of routine exploration of the CBD in cases of patients scheduled for cholecystectomy after an attack of ABP was submitted to question. Ito *et al*[32] investigated this in cases of low risk for choledocholithiasis. The authors included 148 patients without preoperative ERCP, normal and decreasing liver function tests, and normal CBD diameter. They were divided into 2 groups - with or without intraoperative cholangiography. Follow-up didn't find any significant differences between the 2 groups regarding postoperative episodes of acute pancreatitis, cholangitis or changes in liver function tests. Authors concluded that direct CBD exploration could be safely avoided in selected cases of ABP, with low-risk for choledocholithiasis.

ETIOLOGY

Some of the acute pancreatitis cases remain idiopathic even after complete serum biochemistry, ultrasound and CT evaluations. Nevertheless, the aetiology of acute pancreatitis should be determined in at least 80% of cases and no more than 20% should be classified as idiopathic (recommendation grade B)[33]. These represent between 10% and 30% in different series. Some studies suggested that more accurate imaging tests for biliary lithiasis detection may reveal the biliary cause in those cases. In our experience, it also happened that once we introduced in our hospital EUS and ERCP there was a shift between the leading causes for acute pancreatitis between the alcoholic and biliary causes, many of idiopathic pancreatitis being actually biliary ones. Recently, some studies showed that a significant rate of the so-called idiopathic pancreatitis are actually caused by microlithiasis and/or biliary sludge, identified by the presence of cholesterol monohidrate and/or calcium bilirubinate microcrystals in the biliary sediment.

Microlithiasis

Microlithiasis is a viscous precipitate containing mucin, cholesterol and calcium bilirubinate which can obstruct the pancreatic duct. US has a sensitivity of only about 55% in detecting microlithiasis and does not allow for analysis of the chemical composition of bile[34]. This is an important cause of recurrent acute pancreatitis. Though a EUS procedure is diagnostic, with a high sensitivity and specificity[35] a duodenal aspirate or a bile duct aspirate for the microliths[36] at ERCP is confirmatory. In a series of 86 patients[37] with acute pancreatitis, 21 patients had microlithiasis. Six patients were subjected to cholecystectomy and 4 patients to endoscopic sphincterotomy. Fewer recurrences were noted in patients receiving either of the two treatment modalities compared to the group managed conservatively. The treatment protocol would warrant a cholecystectomy in all patients unless contraindicated. In those with a high operative risk, endoscopic biliary sphincterotomy is a safe and viable option[38]. Ursodeoxycholic acid is an alternative in those with bleeding tendencies[39]. Thus, microlithiasis or biliary sludge as a causative aetiology for acute pancreatitis remains controversial and not well understood. Several studies have demonstrated the presence of biliary sludge in as many as 75% of patients with unexplained acute pancreatitis[37]. Bile analysis with microscopic examination is considered the gold standard for diagnosis. Bile can be obtained directly while cannulating the bile duct during ERCP or following CCK stimulation on EGD. ERCP with bile aspiration from the CBD has a reported sensitivity of 83% in detecting microlithiasis[40].

In patients considered to have idiopathic acute pancreatitis, after negative routine work-up for biliary etiology, EUS is recommended as the first step to assess for occult microlithiasis, neoplasms and chronic pancreatitis.

If EUS is negative, rare and uncommon causes should be looked for. MRCP (secretin-stimulated) is advised to identify or rule out rare morphologic abnormalities. If aetiology still remains unidentified, genetic counselling (not necessarily genetic testing) should be considered in order to search for hereditary or other genetic causes[3].

In conclusion, the general algorithm for CBD stone detection starts with anamnesis, serum biochemistry and then TUS, followed by EUS or MRCP. In the end, bile duct microscopic analysis may be performed by bile harvested during ERCP in case of recurrent attacks of ABP and these should be followed by laparoscopic cholecystectomy.

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2014 ADVANCES IN PANCREATITIS

Retroperitoneal disorders associated with IgG4-related autoimmune pancreatitis

Noboru Hara, Makoto Kawaguchi, Keisuke Takeda, Yoh Zen

Noboru Hara, Keisuke Takeda, Division of Urology, Department of Regenerative and Transplant Medicine, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan

Noboru Hara, Division of Molecular Oncology, Department of Signal Transduction Research, Graduate School of Medical and Dental Sciences, Niigata University, Niigata 951-8510, Japan

Makoto Kawaguchi, Division of Diagnostic Pathology, Niigata Rousai Hospital, Niigata 951-8510, Japan

Yoh Zen, Institute of Liver Studies, King's College Hospital, SE5 9RS London, United Kingdom

Author contributions: Hara N designed the study and wrote the manuscript; Takeda K assisted in literature searches and data acquisition; Kawaguchi M performed pathological evaluations; Zen Y was involved in editing the manuscript.

Correspondence to: Noboru Hara, MD, PhD, Division of Urology, Department of Regenerative and Transplant Medicine, Graduate School of Medical and Dental Sciences, Niigata University, Asahimachi 1, Niigata 951-8510, Japan. harasho@med.niigata-u.ac.jp

Telephone: +81-25-2272287 Fax: +81-25-2270784

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Abstract

IgG4-related autoimmune pancreatitis is frequently accompanied by relevant lesions in the genitourinary tract and retroperitoneal organs, which cause various clinical problems, ranging from non-specific back pain or bladder outlet obstruction to renal failure. The diagnosis of IgG4-related retroperitoneal fibrosis requires a multidisciplinary approach, including serological tests, histological examination, imaging analysis, and susceptibility to steroid therapy. Radiological examinations are helpful to diagnose this condition, but surgical resection is occasionally unavoidable to exclude malignancy, particularly for patients with isolated retroperitoneal involvement. Steroid therapy is the treatment of choice for this condition, the same as for other manifestations of IgG4-related disease. For patients with severe ureteral obstruction, additional ureteral stenting needs to be considered prior to steroid therapy to preserve the renal function. Some papers have suggested that IgG4-related disease can affect male reproductive organs including the prostate and testis. IgG4-related prostatitis usually causes lower urinary tract symptoms, such as dysuria and pollakisuria. Patients sometimes state that corticosteroids given for IgG4-related disease at other sites relieve their lower urinary tract symptoms, which leads us to suspect prostatic involvement in this condition. Because of the limited number of publications available, further studies are warranted to better characterize IgG4-related disease in male reproductive organs.

Key words: IgG4; Autoimmune pancreatitis; Retroperitoneum; Genitourinary tract; Management

Core tip: Patients with IgG4-related autoimmune pancreatitis frequently have associated conditions involving genitourinary organs. Since clinical presentations and imaging findings vary among patients, the differential diagnoses are broad. Serum IgG4 elevation is highly sensitive but not entirely specific for this condition, which is one reason why the diagnosis should be established in a multidisciplinary way. Although recent radiological advances have facilitated the effective characterization of IgG4-related retroperitoneal fibrosis, surgical resection is occasionally necessary to exclude malignancies. In addition to steroid therapy, ureteral stenting is required for patients with severe ureteral obstruction. A new concept of IgG4-related prostatitis is being increasingly recognized.

Original sources: Hara N, Kawaguchi M, Takeda K, Zen Y. Retroperitoneal disorders associated with IgG4-related autoimmune

INTRODUCTION

IgG4-related disease has been widely recognized over the last decade[1]. Type 1 autoimmune pancreatitis is a prototypic manifestation of this systemic condition, and investigations of autoimmune pancreatitis led to the establishment of a novel entity, "IgG4-related disease"[2-4]. IgG4-related autoimmune pancreatitis is sometimes accompanied by synchronous or metachronous lesions at other anatomical sites. Previous studies suggested that retroperitoneal fibrosis is the most commonly associated condition outside the pancreatobiliary system in patients with IgG4-related autoimmune pancreatitis[5,6]. Tubulointerstitial nephritis is another well-known manifestation of IgG4-related disease. Other urinary tract organs that can be affected by IgG4-related disease include the renal pelvis and ureter. Interestingly, reproductive-organ involvements such as IgG4-related prostatitis have been also confirmed in male patients[7-12].

In this paper, we review features of retroperitoneal and reproductive-organ manifestations related to IgG4-related autoimmune pancreatitis to promote their better understanding and management. We did not include IgG4-related tubulointerstitial nephritis, as it has already been well described[13,14].

RESEARCH

A PubMed search was performed for articles published until November 2013 using the keywords of IgG4, pancreatitis, and retroperitoneal fibrosis or testis or prostate. We also referred to studies published in Japanese, as many studies on this entity have been conducted in Japan. Written informed consent was obtained from all the patients for case presentation.

IgG4-RELATED RETROPERITONEAL FIBROSIS: GENERAL ASPECTS

An association between serum IgG4 elevation and autoimmune pancreatitis was first reported by Hamano *et al*[15] in 2001. The same group also described a case of IgG4-related pancreatitis complicated by retroperitoneal fibrosis, where abundant IgG4-positive plasma cells were histologically identified[16]. This is the first proven case of IgG4-related retroperitoneal fibrosis in the literature. In 2006, Kamisawa *et al*[17] suggested that IgG4-related pancreatitis and retroperitoneal fibrosis belong to a systemic condition, which is now recognized as IgG4-related disease. Since then, many papers have described IgG4-related retroperitoneal fibrosis, but most of them are case reports.

It is worth emphasizing several aspects of IgG4-related retroperitoneal fibrosis. Firstly, some patients present with isolated IgG4-related retroperitoneal fibrosis with no identifiable extra-retroperitoneal lesions. Secondly, retroperitoneal fibrosis is not always IgG4-related. Only approximately 60% of retroperitoneal fibrosis is IgG4-related. Due to marked overlap in clinical features between IgG4-related and non-related cases, this discrimination is not straightforward without histological analysis[18,19]. Yet, if a patient is younger than 40 years, non-IgG4-related retroperitoneal fibrosis is more likely.

The diagnosis of IgG4-related disease thus requires a multidisciplinary approach, where serological tests, tissue diagnosis, and imaging examination need to be considered. Serum IgG4 elevation is highly sensitive, but not entirely specific for this condition. IgG4 elevations up to twice the upper limit of the normal range (280 mg/dL) in the serum can be seen in a variety of diseases, including both inflammatory and neoplastic conditions. IgG4 elevations of more than 280 mg/dL are highly specific for this condition.

IgG4-RELATED RETROPERITONEAL FIBROSIS: PATHOLOGY

IgG4-related retroperitoneal fibrosis is histologically characterized by massive lymphoplasmacytic infiltration, storiform fibrosis, and obliterative phlebitis (Figure 1)[14,20]. IgG4-positive plasma cells should be diffusely present in inflamed area (> 30 cells/high power field) (Figure 2). The rate of IgG4/IgG-positive plasma cells is at least over 40%, typically over 70%[21]. As IgG4-positive plasma cell infiltration is not entirely specific for this condition, the ratio of them to IgG-positive plasma cells is important to avoid overdiagnosis. Histological findings contradicting a diagnosis of IgG4-related retroperitoneal fibrosis include neutrophilic infiltration, necrosis, discrete granuloma, and necrotizing arteritis.

IgG4-RELATED RETROPERITONEAL FIBROSIS: CLINICAL MANIFESTATION

Clinical presentations of IgG4-related retroperitoneal fibrosis are variable. About a half of the patients are believed to be symptom-free. Patients sometimes describe abdominal or back pain and edema of the lower extremities. Once ureters are blocked, symptoms related to hydronephrosis or renal failure may appear.

The spectrum of imaging features is also wide, including soft tissue masses sometimes involving the ureters or renal pelvis (Figure 3), aortic wall thickening involving adjacent soft tissue (Figure 4), or plaque-like diffuse fibrosis[22-24]. On computed tomography (CT), the lesions exhibit a soft-tissue density. On magnetic resonance imaging (MRI), they show a low to intermediate signal intensity on T1-weighted images and various signal intensity patterns on T2-weighted images according to
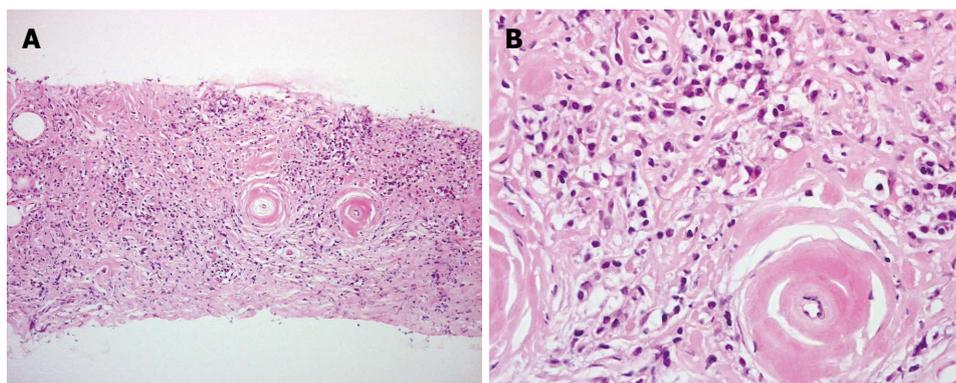
Figure 1 Histological findings with core needle biopsy: Fibrosis and inflammatory reaction with dense infiltration of abundant lymphocytes and plasma cells. A: Low magnification; B: High magnification.
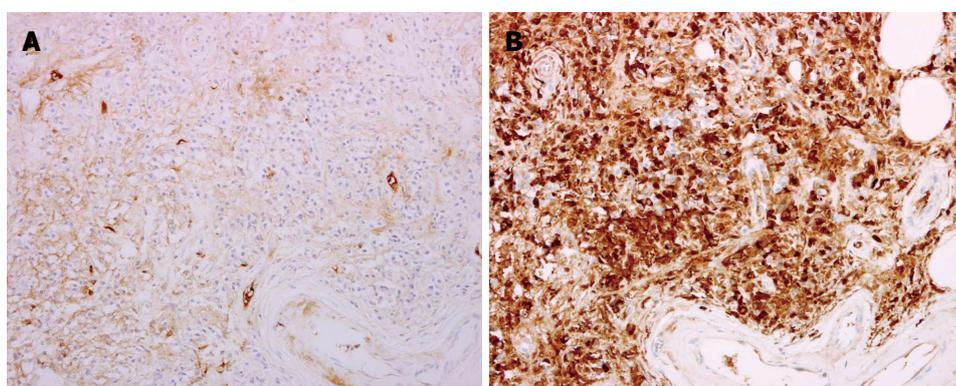
Figure 2 Immunopathological findings: infiltrating cells represent lymphocytes and IgG4-positive plasma cells. A: IgG4 staining; B: IgM staining.
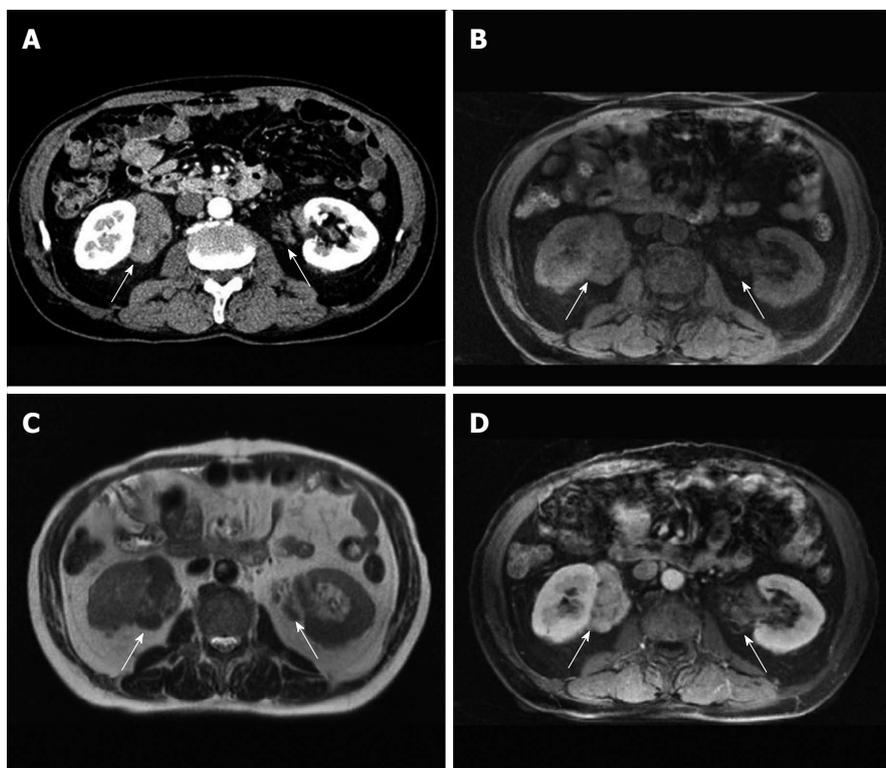
Figure 3 Localized pseudotumors (arrows) in a 67-year-old man with IgG4-related retroperitoneal fibrosis and autoimmune pancreatitis. A: Contrast-enhanced CT; B: T1-weighted; C: T2-weighted; D: Contrast-enhanced MRI.

Figure 4   Pseudotumor spread in the retroperitoneum surrounding the abdominal aorta and vena cava (arrow).

the inflammatory activity (Figure 3). Contrast-enhanced images are useful to estimate the degree of fibrosis and inflammatory activity[25,26].

IgG4-RELATED RETROPERITONEAL FIBROSIS: FUNCTIONAL PROBLEMS

Hydronephrosis related to retroperitoneal fibrosis impairs the renal function, eventually leading to renal insufficiency. Maeta *et al*[27] reported a rare case of acute renal failure due to IgG4-related retroperitoneal fibrosis. Ureteral obstruction was described in 45%-65% of reported patients with IgG4-related retroperitoneal fibrosis[5,6,12,22,24,27-43]. Yet, assuming that many other patients are not reported, how often IgG4-related retroperitoneal fibrosis is associated with hydronephrosis remains unknown. Interestingly, we realized that the left kidney is more commonly affected by this complication in reported cases[5,6,12,22,24,27,29-42]. Tsuboi *et al*[28] reported a 62-year-old man with IgG4-related systemic manifestations, who had left hydronephrosis due to a retroperitoneal pseudotumor. Hart *et al*[12] reported a 67-year-old man who had IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis with hydronephrosis in the left kidney. Two additional cases also had left hydronephrosis[29,30]. Given the fact that most of the patients had IgG4-related autoimmune pancreatitis, the left-sided predominance may be related to the anatomical location of the pancreas. Bilateral hydronephrosis can be also seen at the initial presentation, as described by Miura *et al*[31] and Takenaka *et al*[32]. Hydronephrosis is not common in retroperitoneal fibrosis affecting the renal hilum, as reported by Miyajima *et al*[23].

MANAGEMENT OF IgG4-RELATED RETROPERITONEAL FIBROSIS: PHARMACOLOGICAL THERAPY

The same as for other IgG4-related diseases, steroid therapy is highly effective for IgG4-related retroperitoneal fibrosis[29-31]. However, for patients with ureteral obstruction, how to preserve the renal function is another aspect of treatment. Because of the lack of consensus guidelines for this particular condition, we need to decide on the therapeutic plan (*i.e.*, corticosteroids, ureteral stent) based on the renal function of patients on a case-by-case basis. For patients without severe uremia or fluid retention, oral prednisolone is the most likely treatment of choice. Additional urological intervention such as ureteral stenting may be an option if the obstruction remains even after steroid therapy. Imaging studies involving CT are helpful to assess the effects of steroid therapy[26,37,38].

According to the literature, initial doses vary from 20 mg to 100 mg once daily[28-30,32,33]. A 52-year-old man with IgG4-related pancreatitis and periaortic retroperitoneal fibrosis without urinary tract obstruction was successfully treated with an initial dose of prednisolone of 30 mg[33]. Tsuboi *et al*[28] reported a 62-year-old man with systemic disease, who had left hydronephrosis due to a pseudotumor, successfully managed with prednisolone of 30 mg. Miura *et al*[31] reported an 80-year-old man without pancreatic swelling. His bilateral hydronephrosis was treated successfully with prednisolone of 25 mg. A 51-year-old man with systemic manifestations and bilateral incomplete ureteral obstruction was successfully treated with 50 mg of prednisolone[32]. Both the 67-year-old and 79-year-old men reported by Kikuno *et al*[29] and Nishimura *et al*[30] respectively, were also successfully treated with prednisolone of 30 mg. Low dose prednisolone therapy (initial dose: 0.5-0.6 mg/kg or 30 mg/body daily) with tapering has been the therapeutic standard with encouraging results (recovery rate greater than 90%), the same as for other manifestations of IgG4-related disease[28,31,43].

MANAGEMENT OF IgG4-RELATED RETROPERITONEAL FIBROSIS: UROLOGICAL INTERVENTION

Marked effects of steroid therapy are usually expected in the first couple of weeks. When patients have advanced uremia, severe fluid retention, or ureteral obstruction with symptoms, however, an emergent ureteral stent or nephrostomy is required[27]. Unilateral interventions may be sufficient for recovery from the life-threatening situation. Ureteral stenting is currently the interventional standard. Hart *et al*[12] reported a 67-year-old man who had IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis with hydronephrosis of the left kidney. He underwent ureteral stenting for mild elevation of the serum creatinine level (1.6 mg/dL). Another 74-year-old Chinese man with hydronephrosis received ureteral stenting to relieve ureteral obstruction and associated pain[38]. IgG4-related retroperitoneal fibrosis was thereafter diagnosed in this case. The stent was removed after confirming that pseudotumorous retroperitoneal fibrosis responded well to steroid therapy.

Unilateral nephrostomy needs to be considered for patients with anatomical problems in the lower urinary tract, including severe urethral stenosis and large pros-

Figure 5 Although a ureteral stent was correctly placed into the left renal pelvis (arrow), ipsilateral hydronephrosis did not improve in a 79-year-old woman with IgG4-related retroperitoneal fibrosis.

tatic hyperplasia; IgG4-related autoimmune pancreatitis is most frequently encountered in middle-aged to elderly men (mean age, 59-68 years, 4 to 7.5-fold higher rate compared to women)[4,7,8,17,18]. In fact, we experienced a patient (79-year-old woman) with histologically proven IgG4-related retroperitoneal fibrosis, where ureteral stenting was not sufficient to alleviate ureteral obstruction and renal failure (Figure 5). She subsequently underwent nephrostomy, followed by steroid therapy. Ureteral stents might be constricted by the severe fibrotic process around the ureter.

IgG4-RELATED RETROPERITONEAL FIBROSIS MIMICKING MALIGNANCY

Abe *et al*[41] reported a 39-year-old man, who showed an atypical clinical manifestation. He was diagnosed with a tumor in the left ureter. He underwent segmental ureterectomy, which led to the diagnosis of an IgG4-related periureteral pseudotumor in the distal ureter. Another 75-year old man who metachronously developed IgG4-related autoimmune pancreatitis and retroperitoneal fibrosis was also reported. He initially presented with left hydronephrosis and had surgery for possible ureteral cancer, and an IgG4-related pseudotumor was histologically diagnosed. Ten months later, he was diagnosed with autoimmune pancreatitis, which was successfully managed with prednisolone[34]. These cases suggest that IgG4-related pseudotumorous retroperitoneal fibrosis is difficult to diagnose, particularly when it is the first or an isolated manifestation. Surgical resection is sometimes unavoidable for such patients.

Similar pseudotumorous lesions can develop in the more proximal urinary tract. Yoshino *et al*[42] encountered a 71-year-old man, who had an IgG4-related pseudotumor mimicking renal pelvic cancer in his left kidney. In this case, urine cytology obtained by a retrograde catheter in the renal pelvis was negative for malignant cells. His serum IgG4 level was found to be elevated, and he was successfully treated with prednisolone. The diagnosis of IgG4-related pseudotumorous retroperitoneal fibrosis is

less difficult if patients have other organ manifestations. However, surgery has been conducted even for such patients[33], suggesting that the clinical management of patients with IgG4-related disease requires close coordination between physicians and urologists.

OTHER ASPECTS OF IgG4-RELATED RETROPERITONEAL FIBROSIS

Pipitone *et al*[22] suggested the utility of 18-fluorodeoxyglucose (FDG) positron emission tomography (PET) for the diagnosis and assessment of IgG4-related retroperitoneal fibrosis. This has been supported by additional studies[24,26]. On the other hand, the definite diagnosis of IgG4-related retroperitoneal fibrosis usually requires tissue confirmation, but retroperitoneal biopsies are occasionally problematic due to expected adverse events or technical failure. Sampling error is always a possibility for patients with broad plaque-like lesions. Doe *et al*[40] reported an interesting case: a 77-year-old man was diagnosed with IgG4-related retroperitoneal fibrosis and an elevated serum IgG4 level (398 mg/dL). Retroperitoneal biopsy was not performed because of his marked comorbidity, but lip biopsy revealed the periglandular infiltration of IgG4-positive plasma cells, leading to the diagnosis of IgG4-related disease.

ASSOCIATED CONDITIONS IN THE URINARY BLADDER

There has been no report on urinary bladder involvement in patients with proven IgG4-related disease. Crumley *et al*[44] retrospectively examined biopsy samples of interstitial cystitis from the aspect of IgG4. Interstitial cystitis is a clinical entity previously called painful bladder syndrome, whose etiology and pathogenesis remain undetermined[45,46]. Of 44 cases examined, 4 (9%) showed a significant increase in IgG4-positive plasma cells (greater than 30/hpf) with an IgG4/IgG ratio greater than 0.5. Those patients were characterized by an older age, severer inflammation, and smaller bladder capacity than the remaining 40 IgG4-negative patients. Serological data were not available because of the retrospective nature of the study. Further studies are necessary to conclude whether or not IgG4-related cystitis is a distinct entity.

ASSOCIATED CONDITIONS IN THE PROSTATE

Several case studies on IgG4-related prostatitis have been reported. Patients almost exclusively presented with lower urinary tract symptoms such as dysuria, pollakisuria, urinary urgency, and a feeling of incomplete emptying[9,10]. The clinical presentation is similar to that in common benign prostatic hyperplasia or chronic prostatitis[47,48]. The diagnosis of IgG4-related prostatitis may not be difficult once prostate biopsy is performed; biopsy of the pros-

tate is an established diagnostic routine[49,50]. However, it depends on whether or not urologists, physicians, and pathologists are aware of this condition.

The first case of IgG4-related prostatitis was described in a case series of IgG4-related pancreatocholangitis reported in 2004[4]. Two years later, Yoshimura *et al*[9] described a 65-year-old man, in whom IgG4-related prostatitis was retrospectively diagnosed using IgG4 immunostaining 7 years after he received transurethral resection of the prostate to relieve bladder outlet obstruction. Nishimori and colleagues reported 2 additional cases[10]. Both patients were initially diagnosed with common benign prostatic hyperplasia. One patient with IgG4-related pancreatitis showed the improvement of lower urinary tract symptoms with an alpha-adrenoceptor antagonist that is the first-line agent for men with benign prostatic hyperplasia[48]. He was eventually diagnosed with IgG4-related prostatitis following prostate biopsy, which was performed because of an uptake in the prostate on FDG-PET. The other patient had isolated IgG4-related prostatitis, which was diagnosed by tissue examination of the transurethral resection specimen and elevated serum IgG4 level (473 mg/dL). Interestingly, he also showed FDG uptake in the prostate, while his pancreas was atrophic with no FDG uptake. Zaidan *et al*[51] reported a man with long-standing IgG4-related retroperitoneal fibrosis, who was eventually found to have IgG4-related prostatitis following prostate biopsy. The biopsy was undertaken because of a significant FDG uptake.

Uehara *et al*[52] histologically examined prostate tissue samples obtained from 6 cases, including one radical prostatectomy specimen. This study well addressed the histological characteristics of IgG4-related prostatitis. The histological features are basically similar to those of IgG4-related disease at other sites. Glands are replaced by the inflammatory process, consisting of lymphoplasmacytic infiltration, occasional eosinophils, and irregular fibrosis. Obliterative phlebitis was noted. IgG4-positive plasma cells were diffusely present in inflamed areas.

It is known that serum levels of prostate-specific antigen (PSA) are markedly elevated in men with bacterial prostatitis[49,50], but whether or not IgG4-related prostatitis is associated with an elevated serum PSA remains unclear because of the limited number of cases. Patel and Szostek[53] reported a man with systemic IgG4-related disease, in whom prostatic involvement was confirmed by biopsy. His serum PSA level was within the normal range. Hart *et al*[54] reported a 55-year-old man with IgG4-related pancreatitis and prostatitis (the PSA level was normal: 0.67 ng/mL). Interestingly, his symptoms resolved when he was given a course of oral prednisone for monoarticular gout. In our experience, patients sometimes state that corticosteroids given for IgG4-related disease at other sites relieve their lower urinary tract symptoms, which suggests that IgG4-related prostatitis may be underdiagnosed.

In 2006, Taniguchi *et al*[55] reported a 61-year-old man who presented with retroperitoneal and mediastinal fi-

brosis, and a mass in the left seminal vesicle. IgG4-related disease was diagnosed based on a high serum IgG4 concentration (583 mg/dL) and tissue examination. All lesions responded well to corticosteroids. This is probably the first reported case suggesting IgG4-related disease at this anatomical site.

In summary, men with IgG4-related pancreatitis sometimes present with IgG4-related prostatitis synchronously or metachronously. Lower urinary tract symptoms are common but not specific among elderly men. FDG-PET may be a useful diagnostic modality for IgG4-related prostatitis. Its urological features, such as efficacy of alpha-adrenoceptor antagonists for the alleviation of urinary symptoms and serum PSA elevation, remain unclear.

ASSOCIATED CONDITIONS IN THE TESTIS AND ACCESSORY ORGANS THEREOF

Bösmüller *et al*[11] investigated 3 men (23, 25, and 52 years old) with paratesticular fibrous pseudotumors, and suggested that this condition may be a presentation of IgG4-related disease, although information about coexisting associated conditions in other organs was not provided. Hart *et al*[12] subsequently reported a 67-year-old man who developed a similar condition during observation for IgG4-related autoimmune pancreatitis. Migita *et al*[56] reported a 74-year-old man with bilateral IgG4-related sialadenitis. He had a history of left orchiectomy for a 4-cm paratesticular mass. A retrospective review of pathological slides confirmed an inflammatory mass with the massive infiltration of IgG4-positive plasma cells. de Buy Wenniger *et al*[57] reported a 57-year-old man with IgG4-related pancreatitis, who was found to have IgG4-related orchitis 7 years after pancreaticoduodenectomy. His main symptom was left testicular pain, and the left testis alone was affected. Dieckmann *et al*[45] reported 2 young men with possible IgG4-related orchitis (28 and 18 years old). However, all four patients younger than 30 years (two cases each reported by Bösmüller *et al*[11] and Dieckmann *et al*[45]) showed no other organ involvement, which may challenge the diagnosis in young patients. Information on testicular functions such as testosterone levels and spermatogenesis was not provided in any case reports quoted in this section, and so further studies on endocrinological and functional outcomes of this rare condition are warranted.

CONCLUSION

IgG4-related autoimmune pancreatitis is frequently accompanied by relevant lesions in the retroperitoneum and genitourinary tract, leading to various clinical presentations and imaging abnormalities. Although surgical resection is occasionally unavoidable to rule out malignancy, such incidences will decrease as IgG4-related retroperitoneal fibrosis is more widely recognized and imaging findings are well characterized. We need to decide on the

therapeutic plan (*i.e.*, corticosteroids, ureteral stent) based on the renal function of patients on a case-by-case basis. Increasing evidence suggests that IgG4-related disease can affect the prostate and testis, and further studies are necessary to better understand reproductive-organ involvement of IgG4-related disease.

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2014 ADVANCES IN PANCREATITIS

Diagnosis of autoimmune pancreatitis

Hiroyuki Matsubayashi, Naomi Kakushima, Kohei Takizawa, Masaki Tanaka, Kenichiro Imai, Kinichi Hotta, Hiroyuki Ono

Hiroyuki Matsubayashi, Naomi Kakushima, Kohei Takizawa, Masaki Tanaka, Kenichiro Imai, Kinichi Hotta, Hiroyuki Ono, Division of Endoscopy, Shizuoka Cancer Center, Shizuoka 411-8777, Japan
Author contributions: All author contributed on this work.
Correspondence to: Hiroyuki Matsubayashi, MD, PhD, Chief of Pancreatobiliary Endoscopy, Division of Endoscopy, Shizuoka Cancer Center, 1007, Shimonagakubo, Nagaizumi, Suntogun, Shizuoka 411-8777, Japan. h.matsubayashi@scchr.jp
Telephone: +81-55-9895222 Fax: +81-55-9895692
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Abstract

Autoimmune pancreatitis (AIP) is a distinct form of chronic pancreatitis that is increasingly being reported. The presentation and clinical image findings of AIP sometimes resemble those of several pancreatic malignancies, but the therapeutic strategy differs appreciably. Therefore, accurate diagnosis is necessary for cases of AIP. To date, AIP is classified into two distinct subtypes from the viewpoints of etiology, serum markers, histology, other organ involvements, and frequency of relapse: type 1 is related to IgG4 (lymphoplasmacytic sclerosing pancreatitis) and type 2 is related to a granulocytic epithelial lesion (idiopathic duct-centric chronic pancreatitis). Both types of AIP are characterized by focal or diffuse pancreatic enlargement accompanied with a narrowing of the main pancreatic duct, and both show dramatic responses to corticosteroid. Unlike type 2, type 1 is characteristically associated with increasing levels of serum IgG4 and positive serum autoantibodies, abundant infiltration of IgG4-positive plasmacytes, frequent extrapancreatic lesions, and relapse. These findings have led several countries to propose diagnostic criteria for AIP, which consist of essentially similar diagnostic items; however, several differences exist for each country, mainly due to differences in the definition
of AIP and the modalities used to diagnose this disease. An attempt to unite the diagnostic criteria worldwide was made with the publication in 2011 of the international consensus diagnostic criteria for AIP, established at the 2010 Congress of the International Association of Pancreatology (IAP).

Core tip: Autoimmune pancreatitis (AIP) was first reported in Japan in 1995. Since then, a large series of studies has been documented and the concept of AIP is now recognized worldwide. Two distinct subtypes of AIP occur with different incidences in Asian and western countries. Type 1 is often associated with IgG4-related systemic diseases and shares histological features of lymphoplasmacytic sclerosing pancreatitis. Type 2 is usually not associated with IgG4 abnormality and histologically shows idiopathic duct-centric pancreatitis with granulocytic epithelial lesions. Independent diagnostic criteria had previously been used in individual countries, but international consensus diagnostic criteria were published in 2011.

INTRODUCTION

Autoimmune pancreatitis (AIP) was first documented in 1995 by Yoshida *et al*[1], who reported a case of chronic

pancreatitis that fulfilled the definition of an autoimmune disease[2] with respect to hyperglobulinemia, positive serum autoantibody, and steroid response. In 2001, Hamano *et al*[3] reported increased serum levels of IgG4 in Japanese patients with AIP. This disease is a form of chronic pancreatitis characterized by frequent presentation with obstructive jaundice, simultaneous and/or metachronal occurrences of extrapancreatic lesions, histology of lymphoplasmacytic infiltrates with fibrosis, and a dramatic response to corticosteroids[4-9]. Symptoms, blood test data, and clinical images of the AIP often resemble those of pancreatic cancer (PC)[10-12], malignant lymphoma[1,13], and other types of pancreatitis. Therefore, differential diagnosis must be conducted carefully.

The first diagnostic criteria for AIP were established in Japan in 2002[14], revised in 2006[15], and revised again in 2011(Table 1)[16]. During this period, the concepts of AIP were well recognized worldwide and nationwide diagnostic criteria were proposed in South Korea[17,18], the United States, Germany[19], and Italy[20]. The conditions and methodologies used in each criterion varied; hence, the cases diagnosed as AIP sometimes differed by country. AIP was later revealed to consist of two distinct subtypes: type 1 AIP, which is characterized by histology resembling that of "lymphoplasmacytic sclerosing pancreatitis (LPSP)," and type 2 AIP or "idiopathic ductcentric pancreatitis (IDCP)[21]" with "granulocytic epithelial lesion (GEL)[8,22]". Type 1 AIP is now considered the pancreatic manifestation of systemic organ disorders termed "IgG4-related diseases (IgG4-RD)[23]", while type 2 is usually not associated with IgG4 activity or extrapancreatic lesions other than ulcerative colitis (UC). The proportions of type 1 and type 2 AIP vary substantially in western and eastern countries. Consensus meetings have been held and international criteria were established in Asia in 2008[24], and on a worldwide scale (international consensus diagnostic criteria: ICDC) in 2011 (Tables 2-4 and Figures 1-3)[25]. The ICDC are presently evaluated as the most sensitive and specific criteria for diagnosing AIP[26].

CLASSIFICATION OF AIP

A worldwide survey of AIP[27] indicated that most cases of AIP in Asia fit the histological profile of LPSP, or type 1 AIP, while European and American cases are a mixture of LPSP and idiopathic duct-centric pancreatitis (IDCP)[21,27,28]. The necessity of adequate pancreatic specimens for histology makes accurate diagnosis of IDCP difficult before resection, and this is probably the reason for the limited number of reported cases of type 2 AIP. The two types of AIP also differ in characteristics depending on the geographical distribution, age and gender of the patients, serological findings, association with extra pancreatic lesions, and relapse ratios (Table 5).

Type 1 AIP

Type 1 AIP is histologically characterized as LPSP and is often associated with: (1) abundant lymphoplasmacytic

Table 1 Clinical diagnostic criteria for autoimmune pancreatitis in 2011 by Japan Pancreas Society (JPS-2011)[16]

A: Diagnostic items
  I : Enlargement of the pancreas:
    (a) Diffuse enlargement
    (b) Segmental/focal enlargement
  II : ERP (endoscopic retrograde pancreatography) shows irregular narrowing of the main pancreatic duct
  III: Serological findings
    Elevated level of serum IgG4 ($\geq$ 135 mg/dL)
  IV: Pathological findings: Among (1)-(4) listed below
    (a) Three or more are observed
    (b) Two are observed
    (1) Prominent infiltration of lymphocytes and plasmacytes and fibrosis
    (2) More than 10 IgG4-positive plasmacytes per high-power microscope field
    (3) Storiform fibrosis
    (4) Obliterative phlebitis
  V: Extra-pancreatic lesions: sclerosing cholangitis, sclerosing dacryoadenitis/sialoadenitis/retroperitoneal fibrosis
    (a) Clinical lesions
      Extrapancreatic sclerosing cholangitis, sclerosing dacryoadenitis/ sialoadenitis (Mikulicz disease) or/retroperitoneal fibrosis
    (b) Pathological lesions
      Pathological examination shows characteristic features of sclerosing cholangitis, sclerosing dacryoadenitis sialoadenitis or/retroperitoneal fibrosis
<Option> Effectiveness of steroid therapy
A specialized facility may include in its diagnosis the effectiveness of steroid therapy, once pancreatic or bile duct cancers have been ruled out. When it is difficult to differentiate from malignant conditions, it is desirable to perform cytological examination using an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA). Facile therapeutic diagnosis by steroids should be avoided unless the possibility of malignant tumor has been ruled out by pathological diagnosis.

B: Diagnosis
  I : Definite diagnosis
    (1) Diffuse type
      I a + III/IVb/V (a/b)
    (2) Segmental/focal type
      I b + II + two or more of < III/IVb/V (a/b) >
      or
      I b + II + < III/IVb/V (a/b) > + Option
    (3) Definite diagnosis by histopathological study
      IVa
  II : Probable diagnosis
    Segmental/focal type: I b + II + < III/IVb/V(a/b) >
  III: Possible diagnosis[1]
    Diffuse type: I a + II + Option
    Segmental/focal type: I b + II + Option

When a patient with a focal/segmental image of AIP on CT/MRI without ERCP findings fulfill more than one of III, Ivb and V (a/b) ERP criteria, he/she can be diagnosed as probable AIP only after the negative workup for malignancy by EUS-FNA, and confirmed as definitive one by an optional steroid response. [1]Possible diagnosis: A case may possibly be type 2, although it is extremely rare in Japan. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image.

infiltration with IgG4-positive cells [> 10 cells/high power field (HPF)]; (2) storiform fibrosis; and (3) obliterative phlebitis (Tables 1, 2 and 5). Type 1 AIP frequently occurs in elderly men and is geographically distributed in greater numbers in Asia[29,30] than in western countries[19,20,22,31]. Type 1 AIP is the pancreatic manifestation

Table 2 Diagnosis of definitive and probable type 1 autoimmune pancreatitis using international consensus diagnostic criteria[25]

Diagnosis	Primary basis for diagnosis	Imaging evidence	Collateral evidence
Definitive type 1 AIP	Histology	Typical/indeterminate	Histologically confirmed LPSP (level 1 H)
	Imaging	Typical	Any non-D level 1/level 2
		Indeterminate	Two or more from level 1 (+ level 2 D[1])
	Response to steroid	Indeterminate	Level 1 S/OOI + Rt or level 1 D + Level 2 S/OOI/H + Rt
Probable type 1 AIP		Indeterminate	Level 2 S/OOI/H + Rt

Criterion	Level 1	Level 2
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)	Indeterminate (including atypical[3]): Segmental/focal enlargement with delayed enhancement
D: Ductal imaging (ERP)	Long (> 1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation	Segmental/focal narrowing without marked upstream dilatation (duct size, < 5 mm)
S: Serology	IgG4, > 2 × upper limit of normal value	IgG4, 1-2 × upper limit of normal value
OOI: Other organ involvement	a or b	a or b
	a: Histology of extrapancreatic organs Any three of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration (2) Storiform fibrosis (3) Obliterative phlebitis (4) Abundant (> 10 cells/HPF) IgG4-positive cells	a: Histology of extrapancreatic organs including endoscopic biopsies of bile duct[4]: Both of the following: (1) Marked lymphoplasmacytic infiltration without granulocytic infiltration (2) Abundant (> 10 cells/HPF) IgG4-positive cells
	b: Typical radiological evidence At least one of the following: (1) Segmental/multiple proximal (hilar/intrahepatic) or proximal and distal bile duct stricture (2) Retroperitoneal fibrosis	b: Physical or radiological evidence At least one of the following (1) Symmetrically enlarged salivary /lachrymal glands (2) Radiological evidence of renal involvement described in association with AIP
H: Histology of the pancreas	LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells	LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (> 10 cells/HPF) IgG4-positive cells
Response to steroid (Rt)[2]	Diagnostic steroid trial Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestations	

[1]Level 2 D is counted as level 1 in this setting; [2]Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration; [3]Atypical: Some AIP cases may show low-density mass, pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm); [4]Endoscopic biopsy of duodenal papilla is a useful adjunctive method because ampulla often is involved pathologically in AIP. AIP: Autoimmune pancreatitis; ICDC: International consensus diagnostic criteria; HPF: High power field; LPSP: Lymphplasmacytic sclerosing; OOI: Other organ involvement.

of IgG4-related disease (IgG4-RD)[23,32]; consequently, a variety of systemic lesions with IgG4-positive cells infiltrates develop simultaneously or metachronously, in association with elevated level of serum IgG or IgG4 (> 135 mg/dL) and positive serum autoantibodies. These systemic lesions include sclerosing cholangitis (60%), sialadenitis (14%), retroperitoneal fibrosis (10%), interstitial pneumonitis (8%), and tubulointerstitial nephritis (8%)[4], and many other organs are recognized as possible targets of IgG4-RD or type 1 AIP[5] (Table 6). Response to corticosteroid therapy is usually excellent (97%-98%)[33,34]; however, a high rate of relapse is also observed (56% in 1 year within steroid initiation and 92% within 3 years) (Table 5).

Type 2 AIP

Type 2 AIP is regarded as a specific pancreatic disease, characterized histologically by duct-centric pancreatitis with a GEL[21,22,27,35]. Type 2 AIP patients are more frequently diagnosed in western countries, with a younger age of onset and without gender deviation, compared to type 1[36]. Type 2 AIP occasionally coexists with inflammatory bowel disease (16%-30%)[36,37]. Response to steroids is excellent, as in type 1, but type 2 AIP rarely relapse (Table 5)[37].

Patients with type 2 AIP have no serological markers of autoimmunity. Therefore, the classification of type 2 AIP as a clinical entity of AIP is still debated. Nevertheless, the deposition of C3c and IgG in the basement

Table 3 Diagnosis of definitive and probable type 2 autoimmune pancreatitis using international consensus diagnostic criteria[25]

Diagnosis	Imaging evidence	Collateral evidence
Definitive type 2 AIP	Typical/indeterminate	Histologically confirmed IDCP (level 1 H) or clinical inflammatory bowel disease + level 2 H + Rt
Probable type 2 AIP	Typical/indeterminate	Level 2 H/clinical inflammatory bowel disease + Rt
Criterion	**Level 1**	**Level 2**
P: Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement)	Indeterminate (including atypical[2]): Segmental/focal enlargement with delayed enhancement
D: Ductal imaging (ERP)	Long (> 1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation	Segmental/focal narrowing without marked upstream dilatation (duct size, < 5 mm)
OOI: Other organ involvement		Clinically diagnosed inflammatory bowel disease
H: Histology of the pancreas (core biopsy/resection)	IDCP	
	Both of the following: (1) Granulocytic infiltration of duct wall (GEL) with or without granulocytic acinar inflammation (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells	Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltrate (2) Absent or scant (0-10 cells/HPF) IgG4-positive cells
Response to steroid (Rt)[1]	Diagnostic steroid trial Rapid (≤ 2 wk) radiologically demonstrable resolution or marked improvement in manifestations	

[1]Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration; [2]Atypical: Some AIP cases may show low-density mass, pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm). AIP: Autoimmune pancreatitis; ICDC: International consensus diagnostic criteria; IDCP: Idiopathic duct-centric pancreatitis.

Table 4 Diagnosis of autoimmune pancreatitis-not otherwise specified using international consensus diagnostic criteria[25]

Diagnosis	Collateral evidence (case with only D1/2)
AIP-not otherwise specified	D1/2 + Rt

membrane of the pancreatic ducts and acini suggests an immune complex-mediated destruction of ducts and acini in type 2 as well as type 1 AIP[38].

DIAGNOSTIC CRITERIA OF AIP

Diagnostic criteria, either nationwide[9,16-20] or international[24,25], consist mostly of common diagnostic items such as image findings of the pancreatic parenchyma, pancreatography, and extrapancreatic lesions; serological findings; histology of the pancreatic lesion; and response to steroid therapy (Tables 1-3). The diagnostic items are very similar, but the method or approach for analyzing each finding varies depending on the country. For instance, in Japan[16], endoscopic retrograde pancreatography (ERP) is performed even by general clinicians but is usually precluded in western counties to avoid causing or worsening pancreatitis. In contrast, the Mayo Clinic in the United States[9] routinely performs pancreatic core biopsy for diagnosing AIP. These differences in the methodology seem to reflect the diagnostic criteria or diagnostic algorithm used by individual country[9,16-20].

Pancreatic parenchymal imaging

Focal or diffuse pancreatic enlargement is a common finding in both types of AIP. A dynamic study showed that enhancement of the pancreatic parenchyma is repressed during the arterial to parenchymal phase and is recovered at the portal phase to delayed phase[39]. This enhancement pattern is distinct from that of PC and is applied to contrast-enhanced EUS for the differentiation of AIP and cancer by analyzing time-intensity curves[40,41]. Typically, a linear or band-like structure, depicted as low density by computed tomography (CT) and a hypo-intensity signal by T2-weight magnetic resonance image (MRI), appears at the margin of the enlarged pancreatic parenchyma and is referred to as a "capsule-like rim", reflecting the fibrous tissue[39,42]. Abdominal ultrasonography (US) and EUS show similar findings to those of early chronic pancreatitis, including hyperechoic foci (91%-100%), hyperechoic strands (30%-81%), lobularity (15%-53%), and a hyperechoic wall of the main pancreatic duct (30%) in cases with AIP, and these findings decrease after steroid therapy[33,43]. Ultrasound of typical diffuse-type AIP shows a diffusely enlarged low-echoic pancreas without ductal dilation, or so-called "sausage-like appearance." Elastographic studies have revealed inconsistent results regarding the hardness of pancreatic lesions associated with AIP[44,45].

Pancreatographic imaging

An irregular narrowing of the main pancreatic duct (MPD), but not a complete stenosis or obstruction, is seen in cases of AIP. Nishino *et al*[46] analyzed the differences in ERP findings between AIP and PC, and found a higher prevalence of narrowing of the MPD for ≥ 3 cm of its length and a higher prevalence for the presence of side branches in the narrowed portion of the MPD in the AIP group than in the PC group (*P* < 0.001 and *P* < 0.001,
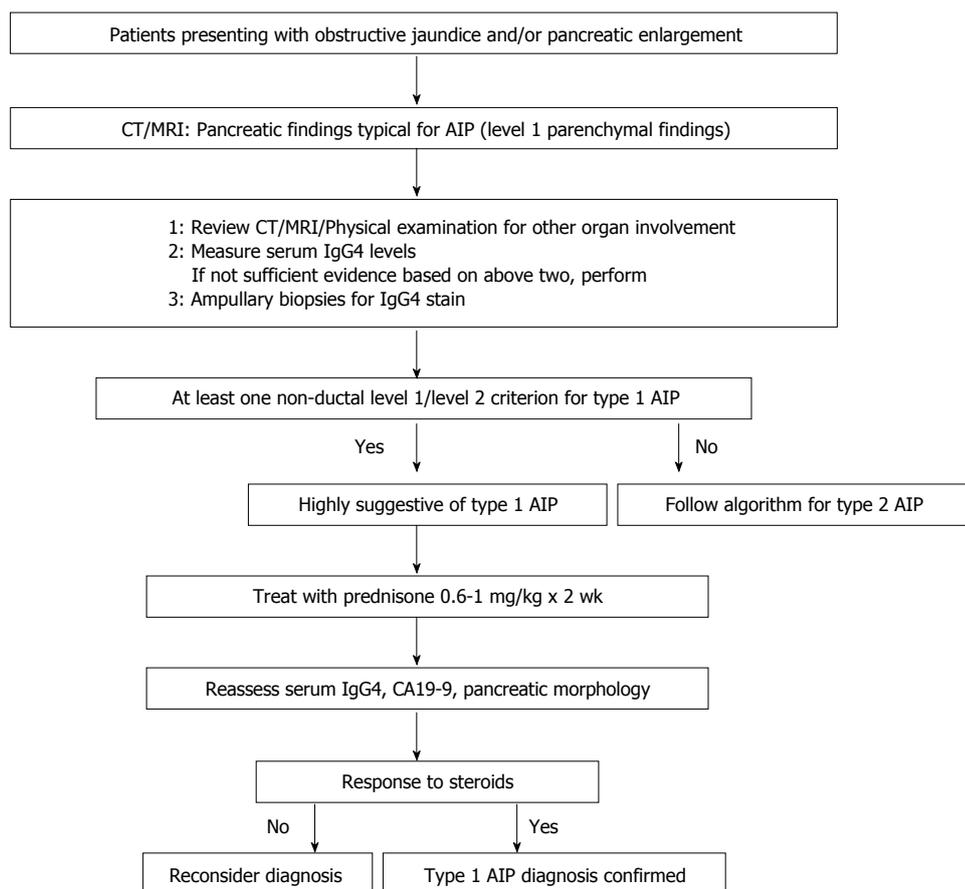
Figure 1 Algorithm of international consensus diagnostic criteria to diagnose type 1 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic enlargement. This schematic drawing shows a flow to diagnose type 1 AIP with typical diffuse enlargement of the pancreas on CT/MRI (level 1 parenchymal findings)[25]. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image.

respectively). In addition, an obvious dilation of the MPD ($\geqslant$ 4 mm) upstream of the lesion was recognized in 87% of the PC cases, but this was seen in only 11% of the AIP cases ($P < 0.001$). The narrowed portion of the MPD is not visualized by magnetic resonance cholangiopancreatography (MRCP)[47]; however, use of ERP is only mandatory in the Japanese criteria (Table 1). Either MRCP or ERP is acceptable in the Korean criteria[17,18] and modality is not specified in the Mayo criteria (HISORt)[9]. The ERCP finding seems to be extremely important in atypical cases[10,33]; for instance, a case that does not show marked shrinkage following steroid therapy[33,48] or a case of PC mimicking[11] or accompanying[12] AIP.

Serology

The most sensitive and specific serum marker for type 1 AIP is IgG4 ($\geqslant$ 135 mg/dL, sensitivity: 86%, specificity to AIP against PC: 96%). However, IgG4 is not actually specific for AIP[5], and elevated serum IgG4 or infiltrations of numerous IgG4-bearing plasma cells have also been reported in cases with PC (10%, 13/135)[49]. Various antibodies appear in the sera of AIP patients, such as anti-lactoferrin antibody, anti-carbonic anhydrase II antibody, antinuclear antibody (ANA), and rheumatoid factor (RF) at respective frequencies of 75%, 55%, 60%, and 20%-30%[50]. The sensitivity of a set of non-specific se-

rum markers (IgG + ANA + RF) (91%) is similar to that of IgG4, but the specificity (61%) is significantly lower than for IgG4[5]. The SS-A (Ro) and SS-B (La) antibodies, which are markers of Sjögren's syndrome, are rarely seen in AIP patients, giving additional grounds for the idea that sclerosing sialadenitis seen in AIP patients is distinct from Sjögren's syndrome.

The level of serum markers is usually correlated with the autoimmune activity and a large number of systemic lesions are more often recognized in type 1 AIP with high levels of serum markers (IgG4, soluble IL2 receptor, *etc.*)[51,52]. Relapse is also often recognized in cases with elevated levels of serum IgG[33] or IgG4[34]. Hence, these serum markers are also applicable to the clinical follow up of patients with type 1 AIP.

Extrapancreatic lesions (other organ involvement)

Extrapancreatic lesions are often associated with type 1 AIP and are correlated with disease activity. The most common extrapancreatic lesion seen in type 1 AIP is sclerosing cholangitis (bile duct), with other typical lesions including dacryoadenitis (lachrymal gland), sialadenitis (salivary gland), interstitial pneumonitis (lung), tubulointerstitial nephritis (kidney), retroperitoneal fibrosis (retroperitoneum), and lymph node lesions at the hepatic hilar portion. Many of reported extrapancreatic lesions
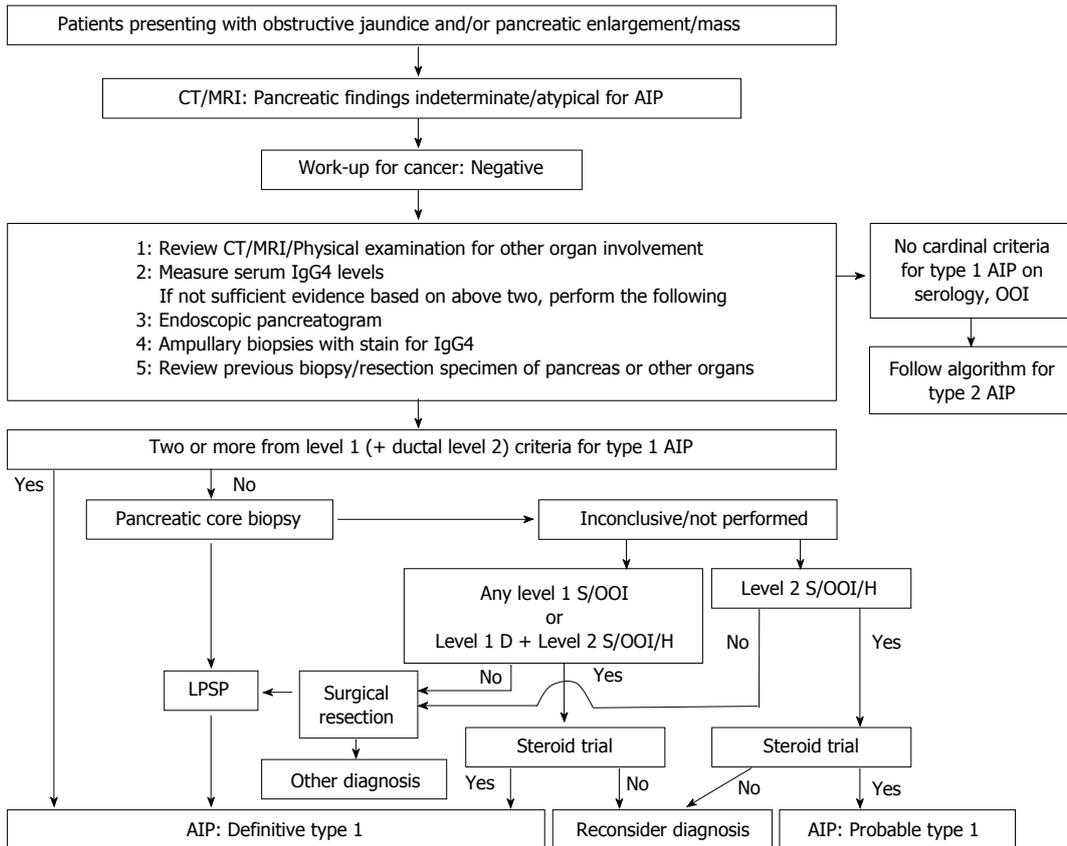
Figure 2 Algorithm of international consensus diagnostic criteria to diagnose type 1 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic mass. This schematic drawing shows a flow to diagnose type 1 AIP with indeterminate or atypical findings of the pancreas on CT/MRI (level 2 parenchymal findings)[25]. AIP: Autoimmune pancreatitis; CT: Computed tomography; MRI: Magnetic resonance image; OOI: Other organ involvement.

are summarized in Table 5 and classified as having close association or possible association with AIP. Representative extrapancreatic lesions have been reported as showing pathological findings similar to the pancreas, including massive lymphoplasmacytic infiltration and fibrosis, obliterating phlebitis, and presence of prominent IgG4 positive plasma cells[7]. These lesions can be detected incidentally in cross-sectional images and whole body imaging such as [18F-]Fluoro-deoxyglucose positron emission tomography (PET)[53,54] and Gallium scintigraphy[55]. These extrapancreatic lesions sometimes confuse the diagnosis; *i.e.*, type 1 AIP is sometimes accompanied by pseudotumor of the liver or lung, mimicking metastases from PC[56]. The occurrence of OOI in AIP patients sometimes causes serious physical conditions, such as loss of consciousness due to swelling of the pituitary gland[57] or hemorrhagic risk due to the decreased platelet numbers caused by autoimmune thrombocytopenic purpura in cases with anticoagulant intake[58].

Histology of the pancreatic lesion
The pancreatic lesion of type 1 AIP histologically shows LPSP with 3 essential features: (1) a lymphoplasmacytic infiltrate surrounding small-sized interlobular pancreatic ducts that does not destroy the pancreatic ductal epithelium; (2) a swirling fibrosis centered around ducts and veins (storiform fibrosis); and (3) obliterative phlebitis

wherein the infiltrate surrounds and obliterates pancreatic veins. Destructive changes to the ducts and acini caused by infiltrating granulocytes are typically absent. Immunostaining reveals abundant IgG4-positive cells (> 10 cells/HPF)[27,31].

Type 2 AIP histology typically shows IDCP (AIP with GELs)[21,27,31], which is a distinct histological pattern from that of LPSP. The predominant interlobular stroma composed of lymphocytes plasma cells and reactive fibroblasts/myofibroblasts seen in type 1 AIP is replaced by the presence of GELs as the most distinctive feature of IDCP. These changes may lead to the destruction and obliteration of the duct lumen, seen in the medium to small-sized ducts and also in the acini. Infiltrates of IgG4-positive plasma cells are scant or absent in IDCP[27,31]. Currently, a definitive diagnosis of type 2 AIP requires histology (Table 3 and Figure 3). This unique histological subtype could be distinguished from type 1 AIP by expert pathologists with high diagnostic ratio (concordances: 60%-100%, multirater kappa: 0.54) using the international consensus histopathological diagnostic criteria[28].

The feasibility of arriving at a histological diagnosis for AIP using endoscopically obtained tissue samples has been argued[59-62]. Several studies demonstrated that tissue samples obtained by endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) enabled histological diagnosis of both type 1[60-62] and type 2[63,64] AIP.
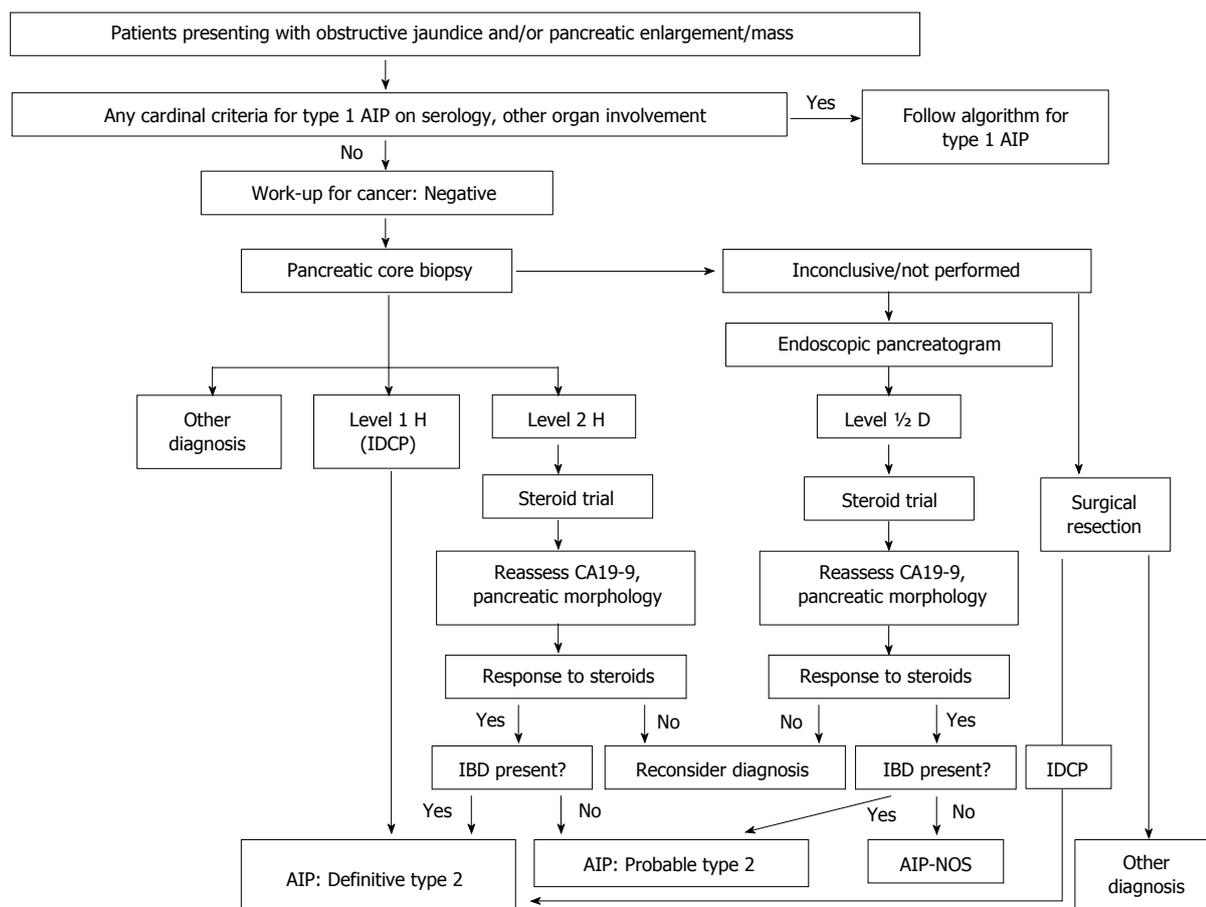
Figure 3 Algorithm of international consensus diagnostic criteria to diagnose type 2 autoimmune pancreatitis in subjects presenting with obstructive jaundice and/or pancreatic mass. This schematic drawing shows a flow to diagnose type 2 AIP with typical/indeterminate (atypical) findings of the pancreas on CT/MRI (level 1 and 2 parenchymal findings)[25]. AIP: Autoimmune pancreatitis; IBD: Inflammatory bowel disease; IDCP: Idiopathic duct-centric chronic pancreatitis.

Exclusion of the pancreatobiliary malignancies

Exclusion of pancreatobiliary malignancies is necessary for the diagnosis of AIP, especially in atypical cases. Today, the diagnosis of pancreatic mass lesions by EUS-FNA provides a sensitivity for detecting PC tissue that exceeds 90% (91%-93%)[59,65,66], making EUS-FNA the most effective tool for excluding pancreatic malignancies. However, core biopsy using a large-caliber needle[60,61,67] may increase the chance of a definitive histological diagnosis of AIP. A Japanese nationwide survey published in 2012[68] reported that histological confirmation was obtained in about 40% of AIP cases by EUS-guided tissue sampling, in 22% by resection, and in 18% by percutaneous biopsy. The choice of suitable modalities for histological evaluation can therefore eliminate non-necessary surgery in a large number of cases.

AIP is often associated with sclerosing cholangitis, which needs differential diagnosis from bile duct cancer. In this sense, periampullary forceps biopsy (and cytology) should be added in cases with biliary stricture, as this method has high sensitivity for confirming cancer tissue in the biliary cancer cases (77%[69,70]-92%[71]).

Response to steroid

Steroid response is seen in 97%-98% of both type 1 and type 2 AIP cases[33,34]; hence, it is considered a useful diagnostic tool. Moon *et al*[72] performed a 2 wk steroid trial on 22 consecutive patients with a pancreatic mass lesions atypical for AIP and used by CT and MRCP/ERCP to determine the steroid response. All 15 patients who responded to steroid were diagnosed with AIP, whereas all 7 patients who did not show a steroid response were confirmed as having PC[72]. We also used abdominal US to analyze the steroid response of the pancreatic lesion of AIP, and we recognized a steroid response (shrinkage of the pancreatic lesion) in 86% of the cases in 2 wk and in 97% after one month[33]. However, one case in this study showed no response by US and CT and required ERCP, which revealed an improvement in the narrowing of the MPD and the occurrence of hilar bile duct stenosis after the withdrawal of corticosteroid[33,48]. Similarly, some cases of AIP fulfill the diagnostic criteria after cessation of steroid[73], so that clinicians need to remain aware of this. Many diagnostic criteria including those for ICDC (Table 2) can include evaluation of a steroid response either in the pancreatic or extrapancreatic lesions[9,17,18,25], but the diagnosis is worrisome when the steroid response is seen only in the extrapancreatic lesions and not in the pancreas.

Today, a "response to steroid" is a commonly evalu-

Table 5 Characteristics of clinicopathological findings in type 1 and type 2 autoimmune pancreatitis

	Type 1 AIP	Type 2 AIP
Geographical distribuition	Asia > United States, Europe	Europe > United States > Asia
Age at presentation	60-70 s	40-50 s
Gender	Male >> Female	Male = Female
Symptoms	Jaundice, Abdominal pain	Jaundice, Abdominal pain
Serology	IgG4, IgG, Autoantibodies	Usually negative
Pancreatic images	Enlarged (focal, diffuse)	Enlarged (focal, diffuse)
Pancreatic histology	LPSP	IDCP with GEL
Extrapancreatic lesions	Sclerosing cholangitis, sialoadenitis, retroperitoneal fibrosis, interstitional nephritis, *etc*.	Inflammatory bowel disease
Steroid response	Excellent	Excellent
Relapse	High rate	Rare

AIP: Autoimmune pancreatitis; LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP with GEL: Idiopathic duct-centritic pancreatitis with granulocyte epithelial lesion.

Table 6 Extrapancreatic lesions associated with type 1 auto-immune pancreatitis

Close association	Possible association
Lachrymal gland inflammation	Hypophysitis
Sialoadenitis	Autoimmune neurosensory hearing loss
Hilar lymphadenopathy	Uveitis
Interstitial pneumonitis	Chronic thyroiditis
Sclerosing cholangitis	Pseudotumor (breast, lung, liver)
Retroperitoneal fibrosis	Gastric ulcer
Tubulointestinal nephritis	Swelling of Papilla of Vater
	IgG4 hepatopathy
	Periaortitis
	Prostatitis
	Schonlein-Henoch purpura
	Autoimmune thrombocytopenia

ated diagnostic item for AIP in almost all diagnostic criteria[9,16-20,25]. However, it had not been included in the previous Japanese diagnostic criteria (2006)[15] in order to avoid simplistic therapeutic diagnosis by a steroid response without exclusion of possible pancreatobiliary malignancies. Clinicians must be careful in making differential diagnoses, and when malignant conditions are difficult to differentiate, pathological examination by EUS-FNA is preferable.

CONCLUSION

AIP is a unique form of chronic pancreatitis consisting of two distinct subtypes and associated with various systemic disorders. An accurate diagnosis can only be obtained when clinicians have a good understanding well on this disease entity and need to make use of diagnostic items including clinical images for pancreatic parenchyma, pancreatography and extrapancreatic lesions, serum markers, histological examinations of the pancreatic lesion, and steroid responses.

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2014 ADVANCES IN PANCREATITIS

Antioxidative phytoceuticals to ameliorate pancreatitis in animal models: An answer from nature

Jong-Min Park, Sooyeon Lee, Mi Kyung Chung, Sung-Hun Kwon, Eun-Hee Kim, Kwang Hyun Ko, Chang Il Kwon, Ki Baik Hahm

Jong-Min Park, Sooyeon Lee, Mi Kyung Chung, Sung-Hun Kwon, Eun-Hee Kim, Ki Baik Hahm, CHA Cancer Prevention Research Center, CHA University, Seoul 135-081, South Korea
Kwang Hyun Ko, Chang Il Kwon, Ki Baik Hahm, Digestive Disease Center, CHA University Bundang Medical Center, Seongnam 463-838, South Korea
Author contributions: Park JM and Lee S performed the majority of experiments; Chung MK, Kwon SH and Ko KH contributed new reagents or analytic tools for this work; Kim EH, Kwon CI and Hahm KB designed the study; Park JM and Hahm KB analyzed the data and wrote the manuscript.
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Correspondence to: Ki Baik Hahm, MD, PhD, Professor, CHA Cancer Prevention Research Center, CHA University, 605 Yeoksam 1-dong, Gangnam-gu, Seoul 135-081, South Korea. hahmkb@cha.ac.kr
Telephone: +82-2-34682869   Fax: +82-2-34682868
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Abstract

Despite enthusiastic efforts directed at elucidating critical underlying mechanisms towards the identification of novel therapeutic targets for severe acute pancreatitis (SAP), the disease remains without a specific therapy to be executed within the first hours to days after onset of symptoms. Although earlier management for SAP should aim to either treat organ failure or reduce infectious complications, the current standard of care for the general management of AP in the first hours to days after onset of symptoms include intravenous fluid replacement, nutritional changes, and the use of analgesics with a close monitoring of vital signs. Furthermore, repeated evaluation of severity is very important, as the condition is particularly unstable in the early stages. In cases where biliary pancreatitis is accompanied by acute cholangitis or in cases where biliary stasis is suspected, an early endoscopic retrograde cholangio-pancreatography is recommended. However, practice guidelines regarding the treatment of pancreatitis are suboptimal. In chronic pancreatitis, conservative management strategies include lifestyle modifications and dietary changes followed by analgesics and pancreatic enzyme supplementation. Recently, attention has been focused on phytoceuticals or antioxidants as agents that could surpass the limitations associated with currently available therapies. Because oxidative stress has been shown to play an important role in the pathogenesis of pancreatitis, antioxidants alone or combined with conventional therapy may improve oxidative-stress-induced organ damage. Interest in phytoceuticals stems from their potential use as simple, accurate tools for pancreatitis prognostication that could replace older and more tedious methods. Therefore, the use of antioxidative nutrition or phytoceuticals may represent a new direction for clinical research in pancreatitis. In this review article, recent advances in the understanding of the pathogenesis of pancreatitis are discussed and the paradigm shift underway to develop phytoceuticals and antioxidants to treat it is introduced. Despite the promise of studies evaluating the effects of antioxidants/phytoceuticals in pancreatitis, translation to the clinic has thus far been disappointing. However, it is expected that continued research will provide solid evidence to justify the use of antioxidative phytoceuticals in the treatment of pancreatitis.

Key words: Acute pancreatitis; Chronic pancreatitis; Severe acute pancreatitis; Antioxidants; Phytoceuticals

Core tip: In this review, the paradigm shift regarding the development of phytoceuticals and antioxidants is introduced following a comprehensive description

of newer information pertaining to the pathogenesis of pancreatitis. Several animal models are discussed with regard to their role in efforts to develop efficient strategies against pancreatitis. Subsequently, newer therapeutic options with an emphasis on nutrients and phytoceuticals are reviewed. Further discussion also focuses on the promise of studies evaluating the effects of antioxidants/phytoceuticals in pancreatitis, the disappointing nature of translation of these agents to clinical settings, and the expected research advances that may support the use of antioxidative phytoceuticals in the treatment of pancreatitis.

INTRODUCTION

Acute pancreatitis (AP) is a relatively common clinical condition, presenting with variable severity from mild and self-limited attacks to severe attacks that contribute to mortality[1]. Severity is associated mechanistically with the underlying pathogenesis of AP which includes pancreatic acinar cell injury in early stages after a local inflammatory reaction, subsequent acinar cell death in the form of apoptosis and necrosis, and the initiation of systemic inflammatory response syndrome (SIRS). An excessive SIRS leads to distant organ damage referred to as multiple organ dysfunction syndrome (MODS)[2]. Recent insights changed a paradigm shift in understanding of AP that intra-acinar trypsinogen activation might lead to early pancreatic injury, but the inflammatory response of AP develops independently driven by early activation of enzyme activation[3]. Whereas, though still effective, the concept that the pancreatic injury is initiated within pancreatic acinar cells subsequent to premature intracellular activation of digestive enzymes and these zymogen activations within acini early during AP was shown to be sufficient to induce AP, finally contributed to the development of chronic pancreatitis[4,5]. Recently, Sah *et al*[6] found that cerulean-induced chronic pancreatitis (CP) did not require intra-acinar activation of trypsinogen, whereas regulation of the inflammatory response by nuclear factor kappa B (NF-κB) might be involved in the pathogenesis of CP. Collectively, these data suggest a need for the development of novel compounds to either block the early activation of pancreatic enzymes or to ameliorate inflammation in order to limit or prevent complications of AP or inhibit the progression to CP or inflammation-associated fibrosis or carcinogenesis. The delay between the onset of pancreatitis and the development of the systemic response makes AP an ideal experimental and clinical model with which to study the role of inflammatory

mediators and to test novel therapies, as the elucidation of the key mediators involved in the pathogenesis of AP will facilitate the development of clinically effective anti-inflammatory therapies[7].

Recent advances in understanding the pathogenesis of pancreatitis-induced SIRS and its complications

AP is an inflammatory disorder, as inflammation not only affects pathogenesis, but also determines the course of the disease from pancreatic acinar cell injury and death to the initiation of SIRS[8]. As excessive SIRS culminates in the primary cause of morbidity and mortality associated with AP, distant organ damage (MODS), it is important to identify the molecules and factors involved in this process. Phospholipase A2 (PLA2), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, CINC/GRO-α, MCP-1, platelet activating factor (PAF), IL-10, CD40L, C5a, ICAM-1, MIP1-α, CCL5 (RANTES), substance P, and hydrogen sulfide (H2S) have all been shown to play critical roles[9]. The systemic effects of AP are similar to those of other conditions such as septicemia, severe burns, and trauma. For instances, AP in its severe form is complicated by MODS, most importantly by pulmonary complications which include hypoxia, acute respiratory distress syndrome, atelectasis, and pleural effusion[10].

Novel pathogenic mechanisms relevant to newer therapeutics: Autophagy, apoptosis, and redox-associated transcriptional activators

Autophagy, the principal cellular degradative pathway for cellular protection, is impaired in pancreatitis and is associated with defective lysosomal function[11]. Although research on autophagy in pancreatitis is now in its early stages, it is hoped that data regarding upstream mechanisms mediating autophagic dysfunction and downstream links to pancreatitis pathologies may provide insights into novel molecular targets and therapeutic strategies for the treatment of pancreatitis[12]. In their detailed explanation of a profound dysfunction of key cellular organelles (lysosomes and mitochondria) in pancreatitis, Gukovsky *et al*[13] described the cause of impaired autophagy in AP and attributed it to inefficient flux resulting from defective lysosomes. Additionally, they suggested that lysosomal dysfunction in pancreatitis could be attributed to either abnormal processing and activation of major lysosomal hydrolases such as cathepsins, or *via* a decrease in pancreatic levels of the key lysosomal membrane proteins LAMP-1 and LAMP-2. NF-κB inactivation is an additional key pathogenic concern in pancreatitis[14]. NF-κB is a nuclear transcription factor responsible for regulating the transcription of a wide variety of genes involved in immunity and inflammation and plays a critical role in pancreatitis as well as extrapancreatic complications and pancreatic cancer[15]. As seen in several animal models of pancreatitis, NF-κB has been critically implicated in either initiation or propagation of pancreatic inflammations, cerulean-induced pancreatitis[16], taurocholate-induced pancreatitis[17], and arginine-induced pancreati-

tis[18]. Relevant to autophagy, NF-κB pathway activation stimulated autophagy during induction of acute necrotizing pancreatitis, after which targeted inhibition of the NF-κB pathway may provide novel therapeutic strategies for reducing the severity of pancreatitis[19]. An additional novel mechanism relevant to newer therapeutics involves apoptosis. To test the hypothesis that preventive apoptosis execution would limit the propagation of necroinflammations in pancreatitis, our group[20] investigated the ability of natural products to induce apoptosis and ameliorate cerulean-induced pancreatitis. Bhatia[21,22] concluded that apoptosis could be a favorable response to acinar cells and that interventions that favor induction of apoptotic, as opposed to necrotic, acinar cell death might reduce the severity of an attack of AP. Aside from pancreatic damage, accelerated acinar cell apoptosis can limit SIRS, as exemplified by honokiol, a low molecular weight natural product similar to *Artemisia*[23]. The pathogenic roles of transforming growth factor-β (TGF-β) signaling[24], H2S bio-gas, and substance P have also come under scrutiny in order to identify potential therapeutic targets. H2S, which plays important physiologic roles in the cardiovascular, central nervous, and gastrointestinal (GI) systems, has been associated with inflammation, especially gastritis and pancreatitis, through vasomodulation and neuromodulation[25,26]. Substance P, a neuropeptide released from nerve endings after binding to neurokinin-1 (NK-1) receptors on the surface of effector cells, plays important roles in several inflammatory states including asthma, immune-complex-mediated lung injury, experimental arthritis, and inflammatory bowel disease, as well as A/CP through increasing microvascular permeability, promoting plasma extravasation, and mediating pain[27]. Bhatia *et al*[28] investigated the interplay between the pro-inflammatory effects of H2S and substance P in a murine model of cerulein-induced AP and suggested that the pro-inflammatory effects of H2S may have been be mediated by the substance P-NK-1 receptor pathway in AP. Lastly, oxygen free radicals in excessively high amounts are all very reactive chemically and can impose a detrimental influence on living organisms by provoking oxidative stress that can damage the pancreas[28].

Recent updates on the pathogenesis of CP relevant to pancreatic inflammation

CP is an inflammatory disease of the pancreas characterized by progressive fibrotic destruction of the pancreatic secretory parenchyma. Genetic studies of hereditary, familial, and idiopathic forms of CP have provided much-needed insight into the pathogenesis of CP. The pivotal role of prematurely activated trypsin within the pancreas in the etiology of CP has been firmLy established based on the identification of gain-of-function missense and copy number mutations in the cationic trypsinogen gene and loss-of-function variants in both the pancreatic secretory trypsin inhibitor and chymotrypsinogen C genes. In particular, variants in the gene encoding carboxypeptidase A1, CPA1, were found to be strongly associated with ear-

ly onset CP[29-31]. Additionally, loss-of-function variants in the cystic fibrosis transmembrane conductance regulator and calcium-sensing receptor genes have also been shown to increase the risk of CP[32]. In addition to these genetic preponderances, necrosis or apoptosis, and inflammation or pancreatic duct obstruction are known to be involved in the pathogenesis of CP. Furthermore, the fibrosing process ultimately leads to progressive loss of the lobular morphology and structure of the pancreas, deformation of the large ducts, and severe changes in the arrangement and composition of the islets. These changes in turn lead to pancreatic insufficiency and predispose patients to changes associated with carcinoma. Irrespective of etiological factors such as heredity, alcohol or nicotine consumption, and nutritional, efferent duct, immunological, and rare metabolic factors, the underlying inflammation and associated subsequent fibrotic destruction of the pancreatic secretory parenchyma are common pathogenic factors in CP that represent targets for prevention through modulation of pancreatic inflammation[33]. Our understanding of CP pathogenesis has improved in recent years through important advances regarding the delineation of mechanisms responsible for the development of pancreatic fibrosis following repeated acute attacks of pancreatic necro-inflammation, also referred to as the necrosis-fibrosis concept[34]. Although steroids can rapidly reduce symptoms in patients with autoimmune CP and micronutrient therapy to correct electrophilic stress is emerging as a promising treatment in the other patients[35], steatorrhea, diabetes, local complications, and psychosocial issues associated with the disease represent additional therapeutic challenges. Such challenges may be resolved in part through intervention with potent anti-inflammatory/anti-oxidative phytoceuticals. In this review, newer therapeutic nutrient-based options and phytoceuticals will be introduced.

ANIMAL MODELS OF PANCREATITIS FOCUSED ON THE DEVELOPMENT OF NEW THERAPEUTICS

Failure to decrease the mortality rate attributable to pancreatitis or improve strategies to prevent CP over the past few decades indicate that current treatment options are limited and predominantly dependent on supportive therapy[36]. Because a key feature of severe AP (SAP) is the presence of extensive tissue necrosis accompanied by inflammatory response syndromes, animal models of AP have become an essential investigative tool for developing potent anti-inflammatory agents. Therefore, a better understanding of the underlying pathophysiology of SAP may lead to more targeted therapeutic options, potentially leading to improved survival. Diverse animal models of AP, from the non-invasive gene knockout and *L*-arginine models as well as the hormone [cerulenin as a cholecystokinin (CCK) analog]-, alcohol-, and immune-mediated-diet [choline deficient, ethionine supplemented,

Table 1 Rodent model to study acute and chronic pancreatitis
Acute pancreatitis
Cerulein ± lipolysaccharide (LPS) or ethanol
Bile salt duct infusion
Duct obstruction ± secretagogues
Diet [choline-deficinet ethionine-supplemented (CDE)]
Cytokines
Coxsackie virus group B (CVB)
Chronic pancreatitis
Cerulein (repeated dosing)
Alcohol
Duct infusion such as trinitrobenzene sulfonic acid or sodium
taurocholate or dibutyltin dichloride
Duct obstruction
Genetic; Cox-2, CFTR, IKK2, LXRb, PERK, TGF-β1
Immunologic
Diet (CDE)
CVB

(CDE)]-induced models, to invasive models including pancreatic duct ligation (PDL), antegrade pancreatic duct perfusion, biliopancreatic duct injection of sodium taurocholate, combination of secretory hyperstimulation with minimal intraductal bile acid exposure, vascular-induced, ischaemia/reperfusion and duct ligation, are available[37] (Table 1). Potential therapeutics can be developed with these animal models, as they share common aspects including the aforementioned pathogenesis of intracellular chemical activation, pancreatic secretion reflux, intracellular production of reactive oxygen species (ROS), and intracellular production of free radicals. As in CP, a special focus on pancreatic duct ligation, repetitive overstimulation with cerulein and chronic alcohol feeding, as well as specific genetic models has been applied[38]. In this review, we will describe some of the animal models used in our efforts to develop efficient strategies against pancreatitis.

Cerulein-induced pancreatitis

Intravenous infusion of the synthetic CCK analog cerulean at a dose of 0.25 μg/kg per hour causes maximal stimulation of pancreatic exocrine secretion[39]. The infusion of supramaximal doses of cerulein (5 μg/kg per hour and 10 μg/kg per hour) induces a significant increase in pancreatic enzymes in blood, as well as interstitial edema and inflammatory cell infiltration that leads to cerulein-induced edematous pancreatitis in rats, mice, dogs, and hamsters. Aside from intravenous infusion, repeated intraperitoneal injections can also be used to induce pancreatitis. In the early phase, large autophagic vacuoles result from fusion of zymogen granules, accompanied by an increase in lysosomal enzyme activity and activation of trypsinogen. However, since the degree of pancreatitis is generally mild, all animals survive the induction of pancreatitis and resolve completely within 6 d after induction. This model of experimental pancreatitis favors the analysis of intracellular events in the early phase of pancreatitis as seen in Figure 1A, which shows edematous pancreatitis, however, the addition of lipopolysaccharide injection or bile duct ligation can to wors-

en simple edematous mild pancreatitis as well as oxidative stress and result in acute hemorrhagic pancreatitis[40-42].

Sodium taurocholate infusion; intraparenchymal or intrapancreatic ductal injection

Paran *et al*[43] are credited with the initial attempt to develop acute necrotizing pancreatitis through intraparenchymal injection of sodium taurocholate in rats. Sodium taurocholate was injected at a dose of 0.3 mL/100 g body weight in concentrations of 5% and 10% into the pancreatic parenchyma of 32 Wistar rats. Early pathological changes observed in the pancreas were focal hemorrhages, parenchymal necrosis, and neutrophil infiltration and at 72 h, the changes observed were acinar necrosis, edema, fibrin deposition and inflammatory cell infiltration. At later time points, changes such as fibrinoid necrosis and fibroblast proliferation were observed[44]. High-pressure infusion of sodium taurocholate into the biliopancreatic duct of rats resulted in significant pancreatic and lung alterations[45]. Taurocholate-induced pancreatitis is therefore a reliable model for severe necrotizing pancreatitis in mice with significantly greater pancreatic damage and systemic inflammatory responses as compared to cerulein-induced pancreatitis and correlate with the clinical observations of multisystem organ failure in AP and early changes in affected organs, suggesting that careful observation should be mandatory in patients with AP in order to institute supportive treatment[46].

L-arginine-induced pancreatitis

In 1984, Hegyi *et al*[47] developed a new type of experimental necrotizing pancreatitis model in rats through the use of a high dose of *L*-arginine administered *via* intraperitoneal administration. This non-invasive model is highly reproducible and produces selective, dose-dependent acinar cell necrosis. Not only is this a good model to study the pathogenic mechanisms of acute necrotizing pancreatitis, but it is also excellent with regard to observing and influencing time course changes of the disease (Figure 1B). Subsequent intraperitoneal injection of 3 g/kg *L*-ornithine caused SAP and higher doses (4 to 6 g/kg) were lethal within hours[48]. Serum and ascitic amylase activities were significantly increased and the increase in pancreatic trypsin activity correlated with the degradation of IκB proteins and elevated IL-1β levels. Oxidative stress in the pancreas was evident from 6 h, making this a simple, noninvasive model of acute necrotizing pancreatitis in rats *via* intraperitoneal injection of 3 g/kg *L*-ornithine. Compared with *L*-arginine, *L*-ornithine was even more effective in inducing pancreatitis. It should be noted that large doses of *L*-arginine produce a toxic effect on the pancreas attributable, at least in part, to the actions of *L*-ornithine.

PDL

AP may be induced by ligating the distal bile duct at the level of the duodenum, which causes the early development of AP, obstructive jaundice and cholangitis in
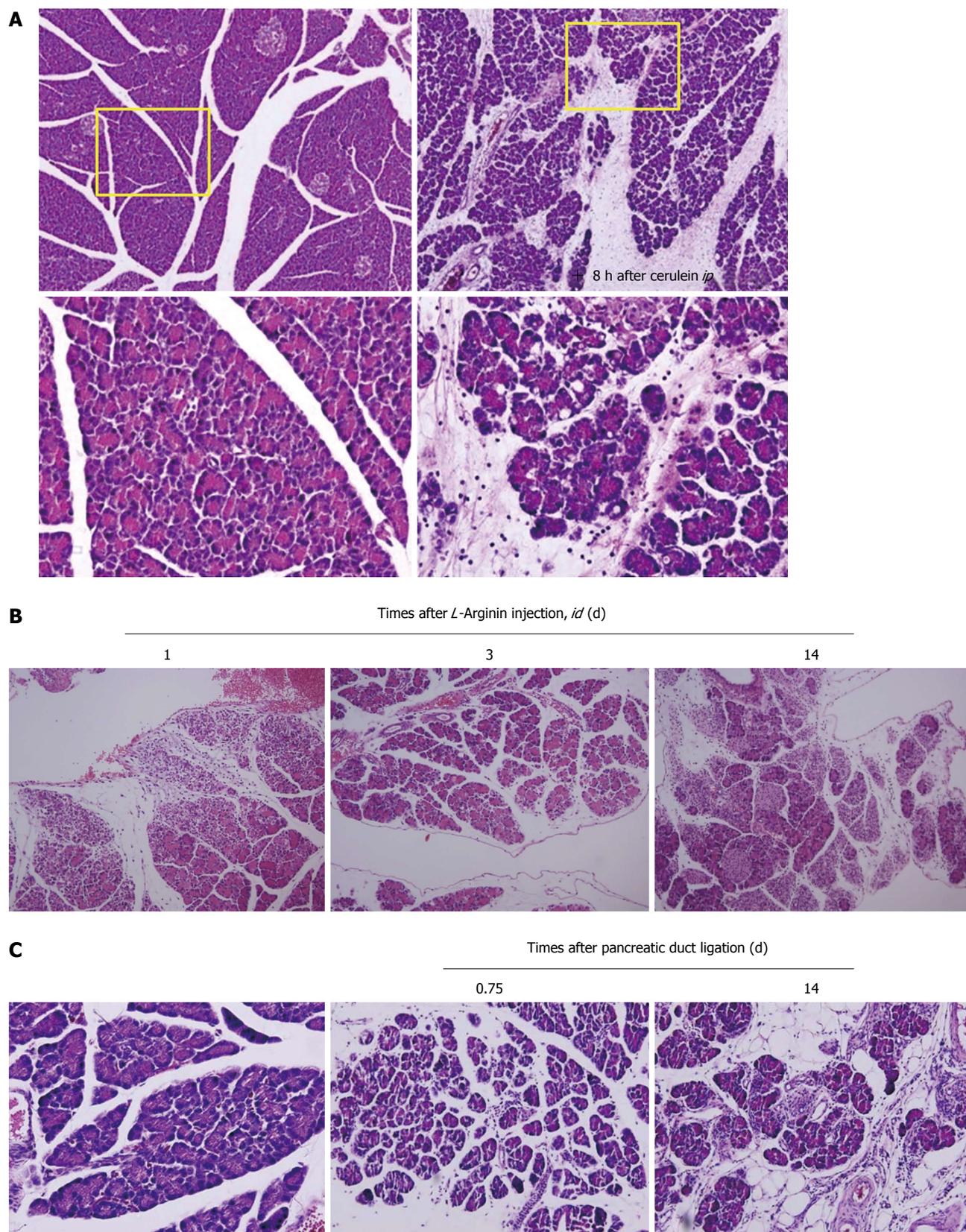
Figure 1 Animal models for pancreatitis. A: Cerulein-induced edematous pancreatitis. Caerulein-induced pancreatitis is a valuable experimental model for studying altered intracellular transport, compartmentation of lysosomal, and digestive enzymes, resulting in edematous pancreatitis. The formation of enlarged secretory vacuoles containing lysosomal and digestive enzymes is paralleled by the activation of lysosomes and degradation of cellular organelles in autophagosomes. On the level of secretory and autophagic vacuoles, activation of serine proteases occurs, which in addition to increasing lysosomal enzyme activities can represent the initial stage for acinar cell destruction and the development of pancreatitis; B: *L*-arginine-induced necrotizing pancreatitis. Parenchymal hemorrhage and widespread acinar cell necrotic changes were noted with *L*-arginine; C: Pancreatic duct ligation-induced pancreatitis. Morphologic examination of the pancreas showed massive interstitial edema, apoptosis, and necrosis of acinar cells with infiltration of neutrophil granulocytes and monocytes 0.75 d after pancreatic duct ligation. Two weeks later after periodontal ligament, the destructed parenchyma with fat replacement as well as some fibrotic changes were seen.

animals. The duct ligation model was developed in an attempt to resemble clinical conditions including gallstone formation, motility disorders of the sphincter, edema and strictures at the papilla, tumors of the papilla, and parasites impacting the terminal biliopancreatic duct. However, surgical ligation of the pancreatic duct alone usually causes only a mild to moderate degree of pancreatitis and has not been successful in inducing SAP. Instead, most laboratory animals developed chronic lesions in the pancreas characterized by atrophy and apoptosis of acinar and ductal tissue without significant necrosis or inflammation. Human CP is characterized by irreversible fibrosis, whereas pancreatic fibrosis in animal models is reversible (Figure 1C). Miyauchi *et al*[49] compared CP with fibrosis in three different animal models, the dibutyltin dichloride model, WBN/Kob rats, and PDL rats, and found that an imbalance between the synthesis and degradation of extracellular matrix molecules or the degree of stimulation over a certain period may lead to pancreatic fibrosis.

CDE diet-induced necrotizing pancreatitis

Female albino mice were fed a choline-deficient diet containing 0.5% DL-ethionine which was lethal within 5 d due to the development of an acute hemorrhagic pancreatitis accompanied by massive fat necrosis throughout the peritoneal cavity[50]. Major findings included the accumulation of zymogen granules, vacuolation due to foci of cytoplasmic degradation, and alterations in the morphology of the zymogen granules (Figure 2A). Pancreatitis appeared to be due to the intraparenchymal activation of zymogens resulting from a synergistic action of choline deficiency with the basic toxicity of ethionine toward the acinar cells of the pancreas. Because this experimental model simulated the acute hemorrhagic pancreatitis with fat necrosis that occurs in humans, it may prove useful for exploring the pathogenesis of severe pancreatitis with SIRS (Figure 2B)[51]. The diet model appears to be a good approximation of severe necrotizing human pancreatitis as well as CP with histological and biochemical similarities. Both the gross and histological appearance of the pancreatic and peripancreatic inflammation, as well as the clinical and biochemical course of diet-induced pancreatitis, resembled human disease and should be suitable for evaluation of potential clinically-applicable drugs[52]. For example, our group developed ND-07, a novel drug candidate with potent antioxidative and anti-inflammatory properties, that effectively prevented necrotizing pancreatitis[53].

Animal models for CP

Since CP is defined as a continuous or recurrent inflammatory disease of the pancreas characterized by progressive and irreversible morphological changes, pancreatitis followed by perilobular and intralobular fibrosis of the parenchyma, calcifications in the parenchyma as well as the formation of pseudocysts[49]. Therefore, animal models of CP are not different from AP models, but need to overcome the acute fatal status according to models, adopting chronic PDL, repetitive overstimulation with

cerulean, chronic alcohol feeding, and chronic caring of *L*-arginine or CDE diet model. However, as seen in Figure 2C, irreversible fibrosis and pancreatic insufficiency following repeated acute attacks of pancreatic necro-inflammation[34], is accompanied.

LIMITATION OF CURRENT PHARMACOLOGIC TREATMENT OF ACUTE AND CHRONIC PANCREATITIS

AP and SAP

Though AP is a disease of variable severity that can lead to significant morbidity and mortality, current management has remained limited to only supportive measures and the treatment of complications. A myriad of pharmacologic therapies targeting various aspects of the underlying pathophysiology have been evaluated and tried over the last few decades, including anti-secretory agents, protease inhibitors, antioxidants, immunomodulators, non-steroidal anti-inflammatory drugs, and prophylactic antibiotics. Only a few of these therapies have demonstrated promise in significantly altering the progression of this disease, and therefore, further studies are necessary to clearly elucidate these benefits in patients at risk for poor outcomes[54]. Regarding pharmacological prevention and treatment of AP, Bang *et al*[55] reported that somatostatin and octreotide inhibited the exocrine production of pancreatic enzymes and may therefore be useful as prophylaxis against post ERCP pancreatitis (PEP). Though the protease inhibitor gabexate mesilate has been used routinely as treatment for pancreatitis in some countries, randomized clinical trials and a meta-analysis have not supported this practice. Recently, the NSAIDs indomethacin and diclofenac have showed some potential as prophylaxis against PEP in randomized studies. Antibodies against TNF-α have been suggested as a potential rescue therapy, however, no clinical trials are being conducted at present[56].

Chronic fibrosing pancreatitis

Because exocrine pancreatic insufficiency has been associated with changes in GI intraluminal pH, motility disorders, bacterial overgrowth, and altered pancreatic gland secretions, drug absorption in patients with CP may be affected by the degree of CP severity[57]. Furthermore, the general health condition of CP patients is often quite poor, as most patients with CP limit their food intake due to the pain caused by eating and in some cases food intake may be more or less substituted with alcohol, tobacco and coffee. However, pancreatic fibrosis is a characteristic feature of chronic pancreatic injury, which is a result of the imbalance between the synthesis and degradation of extracellular proteins. As stellate cells are pivotal cells implicated in the TGF-β induction of collagens, our previous studies confirmed that antioxidant or antioxidative phytoceuticals ameliorated the progression of fibrosing pancreatitis through suppressive actions on pancreatic stellate cells.

A

Times after CDE diet *po* (d)

3 5

B

5 d after CDE

× 40

C
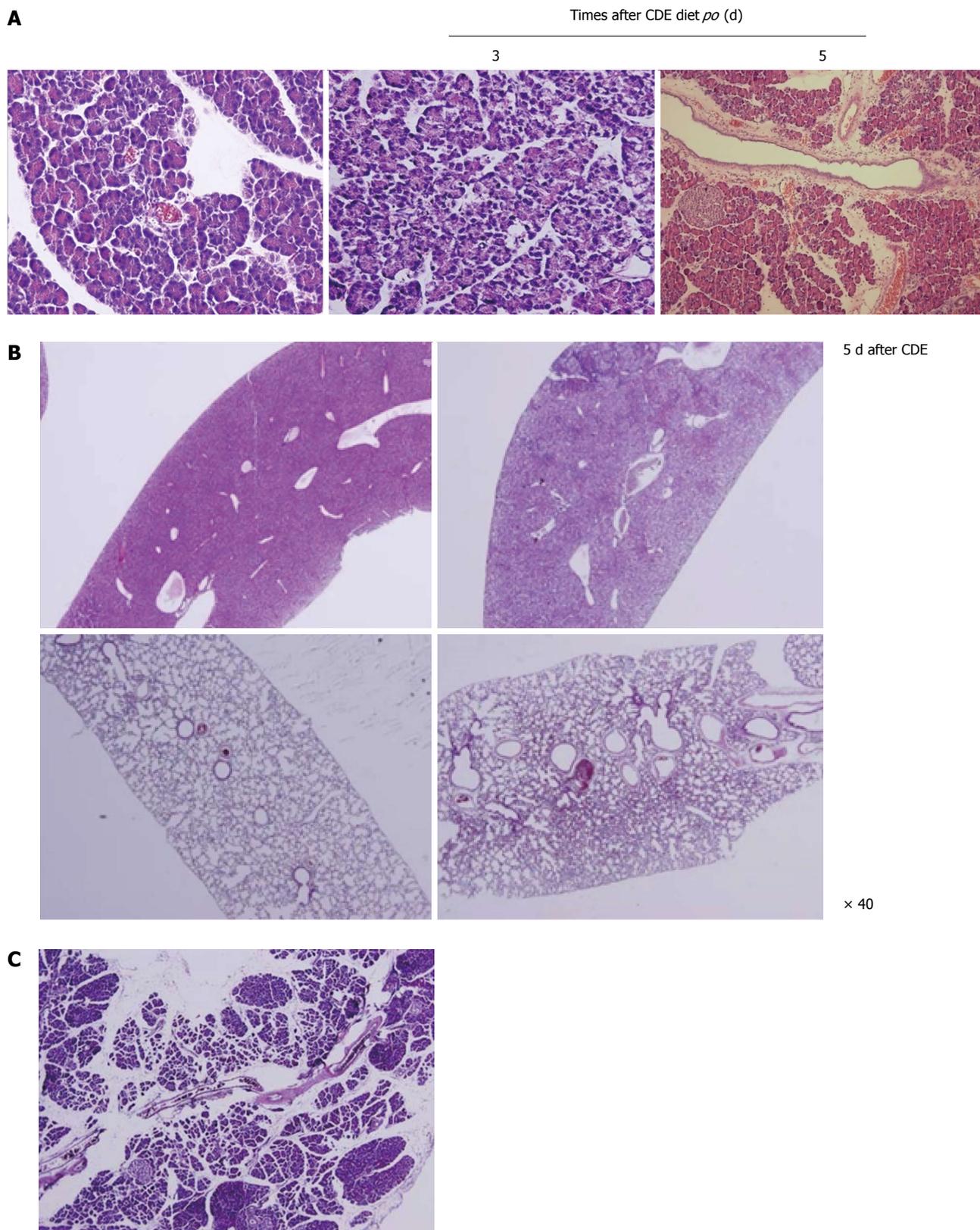
Figure 2 Animal model of choline-deficient, ethionine-supplemented diet-induced necrotizing pancreatitis. A: Choline-deficient, ethionine-supplemented (CDE) diet-induced necrotizing pancreatitis. Massive destruction of pancreatic parenchyma with focal necrotic foci was seen; B: Systemic inflammatory response syndrome hepatic necrosis and pneumonitis was seen; C: Chronic fibrosing pancreatitis was noted 2 mo after CDE diet administration.

APPLICATION OF ANTIOXIDATIVE PHYTOCEUTICALS TO AMELIORATE AP AND CP

Resveratrol

Resveratrol, a natural polyphenolic compound, was first discovered in the 1940s. Although initially used for cancer therapy, it has shown beneficial effects against most cardiovascular, cerebrovascular, and several inflammatory diseases[58]. It is found in diverse forms of plant life, notably berry fruits, has positive effects on metabolism, and can increase the lifespans of various organisms. The effects of resveratrol have been attributed to its capacity to interact with multiple molecular targets involved in diverse intracellular pathways. One of the more well-known resveratrol interactions involves the activation of sirtuins, a class of NAD(+)-dependent deacetylases, and subsequent HDAC inhibition that affects multiple transcription factors and other protein targets[59,60]. The intracellular pathways activated are crucial for anti-oxidant defense, regulation of the cell cycle, mitochondrial energy production, vascular tone, oncogene suppression, and many other phenomena. Meng *et al*[61] investigated whether resveratrol could effectively inhibit the expression of NF-κB activation, alleviate the severity of SAP through its anti-inflammatory effects, and regulate inflammatory mediators. A study by Ma *et al*[62] found that the beneficial outcomes attributable to resveratrol were closely associated with anti-inflammatory, antioxidant, and chemopreventive effects, as well as the inhibition of platelet aggregation, in SAP. Through these effects, resveratrol was able to down-regulate pro-inflammatory cytokines, improve microcirculation, modulate cell apoptosis, and block calcium overload. Additionally, resveratrol inhibited NF-κB activity and reduced concentrations of TNF-α, IL-6 and IL-1β. It also regulated calcium and scavenged ROS capable of extensive tissue damage on extrapancreatic organs[63]. Furthermore, resveratrol has been shown to ameliorate SIRS by improving underlying lung microcirculation dysfunction through decreasing leukocyte-endothelial interactions, reducing blood viscosity, improving the decrease in blood flow, and stabilizing erythrocytes in SAP rats[61] and inactivated intraperitoneal macrophages[64].

Artemisia extracts

Oxygen free radicals (ORFs) mediate an important step in the initiation of experimental AP. Additionally, several clinical findings have implicated OFRs as possible contributors to the pathogenesis of pancreatic fibrosis. To date, there are no studies reporting potential roles for OFRs in the development of CP with the prevention with antioxidants. Yoo *et al*[65] conducted a study designed to establish a mouse model of chronic fibrosing pancreatitis and to prove the involvement of OFRs in CP with fibrosis. Repeated intraperitoneal injection of cerulein provoked significant and severe chronic fibrosing pan-

creatitis after 5 wk. Following treatment with *Artemisia* extracts, the extent of pancreatic fibrosis was significantly decreased, as was the degree of pancreatic inflammation. Furthermore, the level of NF-κB binding activity, which was increased in CP, was significantly attenuated after *Artemisia* extract treatment (Figure 3A). The levels of myeloperoxidase and iNOS activities were also significantly decreased in the *Artemisia*-treated group as compared to the pancreatitis only group. Conversely, cytoprotective proteins such as heat shock protein-70 and metallothionein were significantly increased in the *Artemisia*-treated group. In addition, *Artemisia* decreased the expression of alpha-SMA and type I collagen in cultured pancreatic stellate cells.

Other potential phytoceuticals from nature

There have been published reports describing successful trials demonstrating the beneficial preventive or therapeutic effects of phytoceuticals in diverse animal models of pancreatitis. As examples, rhubarb has been shown to significantly attenuate SAP by inhibiting activation of MAPKs and the expression of inflammatory mediators in taurocholate-induced pancreatitis[66], *Nardostachys jatamansi* has been implicated as potentially protective in cerulean-induced pancreatitis *via* the induction of HO-1 expression[67], and *Curcuma longa* has also been implicated as potentially protective against cerulein-induced AP and pancreatitis-associated lung injury *via* significant attenuation of inflammatory mediators such as IL-1β and TNF-α[68]. Additional examples include the anti-inflammatory roles observed for cannabidiol and O-1602, the ligands of G protein-coupled receptor 55, in cerulein-induced AP in mice[69] and the protective effects of *Scolopendra subspinipes* mutilans water extract in cerulean-induced pancreatitis *via* the deactivation of c-Jun NH2-terminal kinase, p38, and NF-κB and subsequent inhibition of high-mobility group box protein-1[70], Furthermore, attenuation of cerulein-induced AP by apamin, a component of bee venom, or α-pinene, has been observed and attributed to JNK inhibition[71,72] and amelioration of AP by *Dachengqi decoction* has been observed and attributed to regulation of the necrosis-apoptosis switch in the pancreatic acinar cell and rat models[73,74]. Protective effects of three Chinese herbal medicines containing ligustrazine, kakonein, and *Panax notoginsenosides* have been demonstrated on multiple organs in rats with SAP[75] and protective effects of baicalin and octreotide have also been demonstrated on multiple organ injury in SAP[76]. Beneficial pancreatic repair effects have been shown following the use of *Emblica officinalis*, a medicinal plant native to India, or melatonin in *L*-arginine-induced AP in rats[77,78]. An improving effect of pentoxifylline and/or alpha lipoic acid on *L*-arginine-induced SAP has also been described and attributed to antioxidant and anti-inflammatory actions[79]. Other research has shown effects of Korean red ginseng on superoxide dismutase inhibitor-induced pancreatitis in rats through inhibition of NF-κB[80] and the efficacy of *Salvia miltiorrhizae* injection in the treatment of
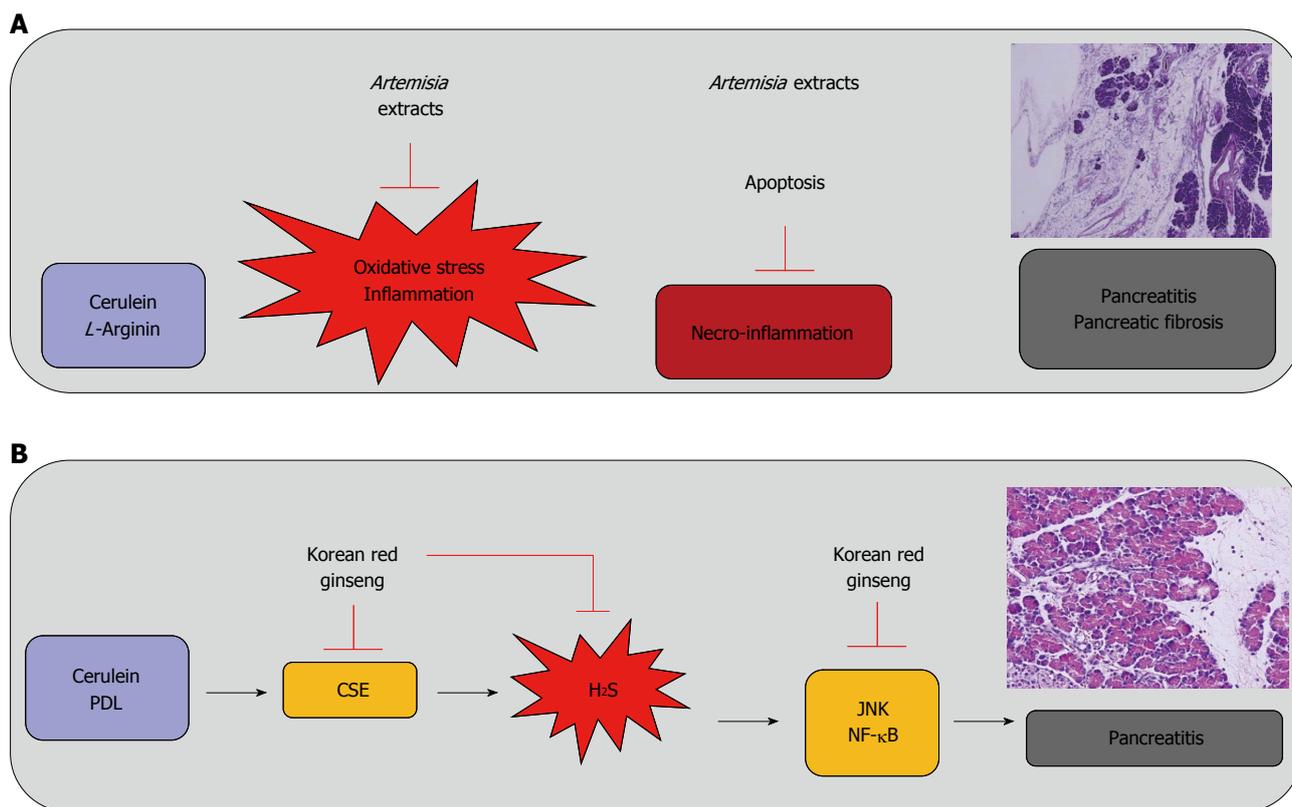
Figure 3 Therapeutic and preventive effect of antioxidative phytoceuticals, Artemisia extract and Korean red ginseng against pancreatitis. A: Therapeutic effect of Artemisia extracts against cerulean or *L*-arginine-induced pancreatitis and chronic fibrosing pancreatitis; B: Korean red ginseng to ameliorate hydrogen sulfide (H2S)-induced pancreatitis. NF-κB: Nuclear factor kappa B; PDL: Periodontal ligament; CSE: Cystathionine γ-lyase.

rats to promote *Bax*-mediated apoptosis in SAP[81].

Antioxidants in the treatment of pancreatitis

Oxidative stress plays an important role in the pathogenesis of both AP and CP. Although its impact has been well documented and has been studied clinically in CP, it is less well defined in SAP. In their study of the pathophysiological aspects of oxidative stress in AP, Hackert and Werner[82] showed that ROS not only participated in the inflammatory cascade, but also mediated inflammatory cell adhesion and consecutive tissue damage. Furthermore, ROS are known to be involved in the generation of pain, an additional important clinical feature of patients suffering from AP. Mechanistically, oxidative stress activates NF-κB, resulting in up-regulation of inflammatory cytokines in pancreatic acinar cells[83]. This mechanism suggests that small-molecule antioxidants may be clinically useful anti-inflammatory agents *via* inhibition of oxidant-induced cytokine production[84]. Similarly, the antioxidant pyrrolidine dithiocarbamate significantly attenuated SAP through inhibition of HMGB1[85] and raxofelast, an inhibitor of lipid peroxidation, significantly reduced NF-κB activation and attenuated cerulean-induced pancreatitis[86]. The potent antioxidant and anti-inflammatory functions of melatonin have also been demonstrated through their ability to ameliorate cerulean-induced pancreatitis by modulating the actions of Nrf2 and NF-κB[87].

KOREAN RED GINSENG TO AMELIORATE PANCREATITIS *VIA* SUPPRESSION OF H2S

Korean red ginseng (KRG) has been reported to reduce the risk of inflammation in diverse organs. In our previous studies[88], we demonstrated significant inhibitory actions of KRG on *Helicobacter pylori*-induced H2S synthesis and the pathogenic connections between H2S synthesis and development of pancreatitis. Therefore, KRG may be a good example of a natural antioxidative phytoceutical for use in ameliorating AP through the inhibition of H2S synthesis. In one of our recent studies that tested the hypothesis that KRG prevents pancreatitis by mitigating H2S generation and pancreatic inflammation, we performed *in vitro* experiments to document the inhibitory effects of KRG on H2S-associated inflammation in pancreatic cells and *in vivo* experiments to document the therapeutic effect of KRG on cerulean-induced and PD*L*-induced AP. KRG was administered at a dose of 200 mg/kg 16 h and 1 h before the first cerulein injection and at a dose of 500 mg/kg 2 h and 4 h after the first cerulein injection by oral gavage. In the mice treated with KRG, pancreatic injuries as evidenced by pancreatic wet weight, histological examinations, serum levels of amylase and lipase, myeloperoxidase activities, serum and pancreatic levels of IL-6, immunohistochemical staining

of F4/80 for infiltrating macrophages, and H2S synthesis, were all significantly ameliorated (Figure 3B). The novel finding that KRG decreased PD*L*-induced hyperamylasemia encouraged us to explore the possibility that KRG pretreatment may prevent ERCP-induced hyperamylasemia. These experiments are ongoing in our clinic.

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Endoscopic prevention of post-endoscopic retrograde cholangiopancreatography pancreatitis

Tae Hoon Lee, Do Hyun Park

Tae Hoon Lee, Division of Gastroenterology, Department of Internal Medicine, Soonchunhyang University School of Medicine, Cheonan Hospital, Seoul 330-721, South Korea

Do Hyun Park, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 330-721, South Korea

Author contributions: Lee TH wrote the manuscript; Park DH developed the concept and revised the manuscript.

Correspondence to: Do Hyun Park, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpagu, Seoul 330-721, South Korea. dhpark@amc.seoul.kr

Telephone: +82-2-30103180 Fax: +82-2-4855782

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Abstract

Post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) is not an uncommon adverse event but may be an avoidable complication. Although pancreatitis of severe grade is reported in 0.1%-0.5% of ERCP patients, a serious clinical course may be lethal. For prevention of severe PEP, patient risk stratification, appropriate selection of patients using noninvasive diagnostic imaging methods such as magnetic resonance cholangiopancreatography or endoscopic ultrasonography (EUS), and avoidance of unnecessary invasive procedures, are important measures to be taken before any procedure. Pharmacological prevention is also commonly attempted but is usually ineffective. No ideal agent has not yet been found and the available data conflict. Currently, rectal non-steroidal anti-inflammatory drugs are used to prevent PEP in high-risk patients, but additional studies using larger numbers of subjects are necessary to confirm any prophylactic effect. In this review, we focus on endoscopic procedures seeking to prevent or decrease the severity of PEP. Among various cannulation methods, wire-guided cannulation, precut fistulotomy, and transpancreatic septostomy are reviewed. Prophylactic pancreatic stent placement, which is the best-known prophylactic method, is reviewed with reference to the ideal stent type, adequate duration of stent placement, and stent-related complications. Finally, we comment on other treatment alternatives, and make the point that further advances in EUS-guided techniques may afford useful PEP prophylaxis.

Core tip: Endoscopic prevention and/or reduction in the severity of pancreatitis (PEP) are considered to be an essential component of appropriate therapy for Post-endoscopic retrograde cholangiopancreatography patients, especially those at high risk. Numerous techniques and drugs have been developed. However, their proven benefits in terms of reducing the severity of pancreatitis are limited. Currently, one popular endoscopic method is prophylactic placement of a pancreatic stent. In this review, we focus primarily on the ideal type of stent, the timing of stent insertion, and the duration of stent placement adequate to prevent PEP. Also, we describe initial cannulation methods including wire-guided cannulation and precut fistulotomy (infundibulotomy), and the alternative techniques of percutaneous biliary drainage and recently emerging endoscopic ultrasonography-guided methodology.

INTRODUCTION

Among complications of endoscopic retrograde cholangiopancreatography (ERCP), post-ERCP pancreatitis (PEP) is the most common, and the clinical course may be downhill. The prevalence of PEP depends on several factors, including the case mix, the thoroughness of follow-up evaluation, the PEP definition used, patient susceptibility factors, the type of instrumentation used, and the skill of the endoscopist[1-4]. PEP occurrence is variable, developing after 1%-40% of all procedures, but typical PEP rates have been reported to range from 5%-15% in most prospective studies with unselected patients. Moreover, pancreatitis of severe grade is very rare, occurring after 0.1%-0.5% of ERCPs[1-6].

Currently, the well-known risk factors for PEP include endoscopic papillectomy, sphincterotomy (including precut or pancreatic sphincterotomy), sphincter of Oddi dysfunction (SOD), younger age, female sex, balloon dilation of an intact biliary sphincter, a previous history of PEP, difficult cannulation or prolonged attempts to cannulate, repeated injection of pancreatic contrast medium, and acinarization[1-11]. These risk factors can be divided into patient-related factors, procedural factors, and operator-related factors.

Although, ideally, PEP should be prevented, complete prevention may be impossible, and decreasing the severity of PEP may be a more realistic goal. Patient risk stratification prior to ERCP, adequate selection of patients using noninvasive diagnostic imaging methods such as magnetic resonance cholangiopancreatography (MRCP) or endoscopic ultrasonography (EUS), avoiding unnecessary procedures, pharmacological prevention and treatment, and the use of various endoscopic techniques to minimize complications, should all be considered. The presence of patient- or procedure-related risk factors allows possible complications to be predicted with reasonable accuracy. Thus, careful patient selection for high-risk and endoscopic procedures, conducted by experienced endoscopists, may reduce procedure-related complications. Pharmacotherapy has also been used widely in efforts to prevent PEP, but the results are inconclusive. Several pharmacological prophylactic treatments have been suggested; these include rectal diclofenac, octreotide, prednisone, and allopurinol[12-17]. Effective prophylaxis of PEP has been demonstrated only using rectal diclofenac or indomethacin[12,15]. However, larger-scale multicenter studies of non-steroidal anti-inflammatory drugs (NSAIDs), with consideration of racial and/or geographical differences, are necessary to confirm any prophylactic effect of PEP. Also, it remains unclear whether NASIDs act synergistically with other prophylactic interventions including pancreatic stenting; this topic requires further work.

In this literature review, we focus on endoscopic aspects of PEP prevention or reduction in severity. We describe primary cannulation techniques including initial wire-guided cannulation, the use of precut fistulotomy (infundibulotomy) and transpancreatic septostomy when cannulation is difficult or as early rescue cannulation

techniques, and prophylactic pancreatic stent (PS) placement during the procedure. Also, we mention alternatives, including percutaneous transhepatic biliary drainage (PTBD), and the possibilities afforded by further advances in EUS-guided biliary drainage techniques.

DEFINITION AND MECHANISMS OF POST-ERCP PANCREATITIS

Cotton *et al*[8] reported a consensus classification of ERCP-related complications. In the cited report, PEP was defined as a clinical syndrome, consistent with acute pancreatitis, associated with a serum amylase level at least three times the normal value, measured more than 24 h after the procedure, and requiring hospital admission or prolongation of planned admission. The severity of PEP is based primarily on the length of hospitalization. Mild PEP is defined as the need for hospital admission or prolongation of planned admission for up to 3 d; moderate PEP is defined by the need for hospitalization for 3-to-10 d; and severe PEP is defined by hospitalization for more than 10 d, or development of significant complications.

The underlying pathogenesis of PEP is thought to be multifactorial, and remains unclear, but numerous mechanisms of PEP induction have been proposed. These include difficult biliary access caused by biliary sphincter hypertension, repeated inadvertent pancreatic duct cannulation and contrast injection, secondary prolonged papillary edema caused by mechanical injury attributable to difficult papillary manipulations, and thermal injury caused by sphincterotomy[18,19]. Thermal injury may be caused by the electrocautery current applied during biliary or pancreatic sphincterotomy, endoscopic papillectomy, or ablation of neoplastic lesions in the region of the ampulla of Vater. Obstructions in the outflow of pancreatic juice may be caused by mechanical injury to the papilla and pancreatic sphincter attributable to use of instrumentation to manipulate the papilla. Chemical or allergic injury may be caused by instillation of contrast medium into the pancreas. Hydrostatic injury may occur after contrast injection into the pancreatic duct or infusion of water or saline through manometry catheters. Enzymatic injury may result from intraluminal activation of proteolytic enzymes as a result of introduction of foreign substances into the pancreatic duct. Infection may also play a role, after pancreatic instillation of flora from the intestine or from contaminated endoscopes or accessories. The results of all of these problems are varied, and include mechanical, chemical, and hydrostatic injury; and infection, triggering premature intracellular activation of proteolytic enzymes, which in turn causes further damage and stimulates local inflammation, as indicated by increased cytokine levels (those of interleukins 1, 6, and 8). If inflammation is severe, a systemic inflammatory response with multi-organ involvement may be activated[1,20,21].

Most strategies for preventing PEP, or decreasing the severity of this condition, have sought to interrupt a step of the inflammatory cascade before, during, or after

ERCP. Endoscopic prevention of PEP seeks to remedy the obstruction of pancreatic outflow caused by the various factors described above.

WIRE-GUIDED CANNULATION

Basic catheterization accompanied by contrast injection was the first cannulation technique developed in the era of ERCP cannulae, and probably remains the most widely used initial cannulation method for ERCP. However, when the first attempts at contrast injection fail, a guidewire may be used as a crossover method to facilitate selective biliary access and to reduce complications caused by prolonged cannula manipulation or contrast injection into the pancreatic duct. Of procedure-related factors, selective cannulation of the common bile duct (CBD) *via* insertion of a guidewire may cause fewer complications than do conventional methods (which use contrast injection to access the bile duct). Ideally, accessing the bile duct with the aid of a guidewire may reduce traumatic injury to the pancreatic duct and papilla, and avoid the buildup of hydrostatic pressure associated with contrast injection, thereby reducing development of ERCP-related pancreatitis[22-26].

Endoscopic technique

Technically, wire-guided cannulation is simple. Usually, a guidewire tipped with a hydrophilic substance, 0.035 or 0.025 inch in diameter, is preloaded into a pull-type papillotome. Next, the papillotome is oriented in the 11-to-12 o'clock position on the papilla, and bent to ensure correct alignment with the axis of the bile duct. In the direct contact method, after minimal insertion (2-3 mm) of the pull-type papillotome into the ampulla, the guidewire is carefully advanced through the CBD under fluoroscopic guidance until it is seen to enter the bile duct. It is also possible to attempt selective cannulation using the slightly (2-3 mm) protruding guidewire on the papillotome to make gentle contact with the papillary orifice. This non-contact method may seek to avoid direct injury caused by contact with the cannula or papillotome. If the pancreatic duct is entered, the guidewire is simply withdrawn and attempts are made to redirect it toward the CBD[22]. However, neither an adequate extent of guideline insertion nor the time that should be permitted for pancreatic duct insertion of the guidewire including retrials, has yet been defined. If unintentional pancreatic duct insertion occurs three-to-five times, it is appropriate to consider switching to another method, such as double-guidewire-induced cannulation, prophylactic PS insertion followed by a precut, transpancreatic septostomy, or early precut fistulotomy, to minimize complications. A precut following prophylactic PS placement may optimally decrease the frequency or severity of PEP, in contrast to use of early precut fistulotomy or double-guidewire-induced cannulation only although these techniques may improve the success rate of selective biliary cannulation.

Clinical outcomes

The PEP-protective effects of wire-guided cannulation remain controversial. In the study by Lella *et al*[25], no patient in a cohort of 200 randomly selected for bile duct cannulation using a soft polytetrafluoroethylene-tipped guidewire (tipped with Teflon; DuPont, Wilmington, DE) developed pancreatitis (0% in the guidewire group *vs* 4.1% in the control group, $P < 0.01$). The cited authors concluded that wire-guided cannulation reduced the frequencies of pancreatic injuries by preventing unintentional injection of contrast media into the main pancreatic duct or the papilla *per se*. However, the authors did not assess PEP frequency with respect to the difficulty of CBD cannulation (the number of cannulation attempts made). Artifon *et al*[23] also showed that use of the guidewire technique for bile duct cannulation lowered the frequency of PEP (8.6% in the guidewire group *vs* 16.6% in the conventional group, $P = 0.02$). The cited authors assessed the difficulty of CBD cannulation, and the numbers of unintentional pancreatic duct cannulations, and concluded that the reduction in PEP was mainly attributable to prevention of injection of contrast media into the pancreatic ducts. The guidewire technique reduced the risk of pancreatitis by facilitating cannulation, by potentially limiting papillary trauma, and by reducing the need to conduct precut sphincterotomies. Although the ranges of cannulation attempts were given as 0 to 3, 4 to 6, and 7 to 10, the investigators did not report the frequencies of PEP development in these subgroups (thus by number of attempts). Even when soft wires tipped with hydrophilic material are used in cannulations, difficult wire passage or frequent pancreatic manipulations may cause injury to the papilla, increasing the risk of PEP. Another randomized study by Lee *et al*[22] showed that wire-guided cannulation reduced PEP development. Totals of 3 patients [2%; 1 mild, 1 moderate, 1 severe (in terms of disease)] in the wire-guided cannulation group and 17 (11.3%; 14 mild, 2 moderate, 1 severe) in the conventional group developed PEP ($P = 0.001$). However, the study population may have been a low-risk cohort. Only seven patients with suspected SOD were included. Among patients with SOD, PEP is a well-recognized complication, occurring at frequencies of 10%-20%[8,19]. SOD independently increases the risk of PEP because of hypersensitivity of the papilla to trauma or an increase in hydrostatic pressure on the main pancreatic duct[3,8,27]. On the contrary, in the study by Vandervoort *et al*[5] guidewire- or sphincterotome-mediated cannulation seem to have been used as rescue methods in high-risk patients who failed conventional cannulation. This explains why the PEP rate was higher when guidewire cannulation was used. Thus, in the cited work, PEP was more frequent in the wire-guided cannulation group (10.2% after wire-guided cannulation *vs* 6.1% after conventional cannulation, $P = 0.04$). However, a recent meta-analysis of the data of five randomized controlled trials showed that the wire-guided technique increased the primary cannulation

Table 1 Prospective randomized trials of wire-guided cannulation to reduce the incidence of post- endoscopic retrograde cholangiopancreatography pancreatitis

	n	Design	Pancreatitis (*n*)/accidental PD (*n*) (WGC *vs* CC)[1]	Post-ERCP pancreatitis *n/n* (%)		*P* value
				WGC	CC	
Lella *et al*[25]	200/200	Prospective/Randomized	0/82, 5/113	0/197 (0)	8/195 (4.1)	< 0.01
Artifon *et al*[23]	150/150	Prospective/Randomized	0/27, 4/21	13/150 (8.6)	25/150 (16.6)	0.02
Bailey *et al*[24]	202/211	Prospective/Randomized	NA	16/202 (7.9)	13/211 (6.2)	0.48
Katsinelos *et al*[26]	167/165	Prospective/Comparative	NA	9/167 (5.4)	13/165 (7.9)	0.37
Lee *et al*[22]	150/150	Prospective/Randomized	2/39, 8/44	3/150 (2)	17/150 (11.3)	0.001
Mariani *et al*[28]	678/571	Prospective/Comparative	15/99, 8/95	35/678 (5.2)	25/ 571 (4.4)	0.60
Kawakami *et al*[29]	199/201	Prospective/Randomized[2]	NA	8/199 (4.0)	6/201 (2.9)	NS

[1]The number of post-ERCP pancreatitis following accidental PD injection or cannulation in CC and WGC group; [2]Multicenter RCT with a 2 × 2 factorial design. 0/82 *vs* 5/113, *P* = 0.08; 0/27 *vs* 4/21, *P* = 0.05; 2/39 *vs* 8/44, *P* = 0.09 by Fisher's exact test. PD: Pancreatic duct cannulation or contrast injection; WGC: Wire-guided cannulation; CC: Conventional cannulation; NS: Not significant; NA: Not available.

rate and reduced the risk of PEP compared to use of the standard contrast-injection method. Pooled analysis of PEP rates in wire-guided cannulation groups compared to those in groups treated using standard methods yielded an OR of 0.23 (95%CI: 0.13-0.41). Also, use of the wire-guided technique was associated with a significantly higher primary cannulation rate (OR = 2.05; 95%CI: 1.27-3.31). Although the meta-analysis included a relatively small number of studies, each work employed different cannulation difficulty criteria (involving cannulation times or numbers of attempts made), and, indeed, some studies did not define their criteria. Three well-designed studies using wire-guided cannulation techniques showed that use of such cannulation could reduce the development of PEP[22,23,25]. However, other recent reported studies have yielded contrary results. Mariani *et al*[28] found that the PEP rates in high- and low-risk patients did not differ between wire-guided cannulation and contrast injection groups (5.2% *vs* 4.4%). In a multicenter randomized study performed by Kawakami *et al*[29], it was also shown that wire-guided cannulation did not reduce the incidence of PEP compared with use of a conventional method (Table 1). In both studies, trainees conducted (some) procedures. When used as an initial cannulation method, wire-guided cannulation seems to shorten cannulation times, as revealed in numerous studies, but any benefit in terms of reducing PEP development is now controversial.

The mechanisms by which guidewire cannulation reduces PEP risk remain uncertain. In the meta-analysis of Masci *et al*[30], several technical issues, including multiple contrast injections into the pancreatic duct, difficult cannulation, precutting, pancreatic sphincterotomy, and balloon dilatation of the sphincter of Oddi, were identified as risk factors for PEP. Notably, the definition of "difficult" cannulation is imprecise, being both subjective and varying among studies. In the report on wire-guided cannulation by Lee *et al*[22], the definition used was failure to achieve biliary access after attempting to do so for 10 min, or after more than five unintentional pancreatic cannulations. Artifon *et al*[23] defined cannulation as difficult when 7-10 attempts were required to ultimately achieve cannulation. Recent studies suggest that the guidelines

for difficult cannulation should be stricter. Large, well-performed, randomized controlled studies aiming to establish cannulation difficulty criteria are needed to resolve these controversies. Also, wire-guided cannulation may not prevent PEP in patients with suspected SOD and who are subjected to unintentional pancreatic duct guidewire cannulation. In high-risk patients, such as those with SOD, repeated unintentional pancreatic duct guidewire cannulation may trigger PEP caused by mechanical trauma or increases in hydrostatic pressure attributable to repeated introduction of a guidewire into the main pancreatic duct. In instances of unintentional pancreatic duct guidewire cannulation, therefore, wire-guided cannulation followed by temporary placement of a PS may be preferred over wire-guided cannulation alone to prevent increases in pancreatic enzyme levels and to reduce the frequency or severity of PEP in high-risk patients[5,22,31,32].

In summary, primary wire-guided cannulation in experienced hands can reduce cannulation time and facilitate successful biliary access, and may reduce the frequency and/or severity of PEP. However, more large-scale comparative studies that consider race, high-risk status, and operator experience, are required to confirm the existence of any prophylactic effect.

PRECUT SPHINCTEROTOMY

Pros and Cons

Precut sphincterotomy is an essential rescue technique in instances of difficult biliary cannulation. Irrespective of the technique used, the initial success rates of precut sphincterotomy have previously been reported to be as high as 90% during the first attempt, with success rates of 95%-99% following second attempts conducted 48-72 h later after edema and inflammation had subsided. In precut methods, various techniques including needle-knife sphincterotomy with or without PS guidance, fistulotomy (infundibulotomy), and transpancreatic sphincterotomy, are used, although few data are available to aid in the selection of a procedure[33-40]. The overall complication rates after precut sphincterotomy have been reported to vary from 1.9% to 34%, compared to rates of 7%-14%

with conventional sphincterotomy. PEP is the most common and serious complication; the rates range from 2.1% to 14.9%, compared to the 1%-10% associated with conventional sphincterotomy[3,33-46].

Although precut sphincterotomy may be an effective rescue technique, such sphincterotomy using a needle-knife has been directly implicated as a primary cause of PEP. Therefore, this technique has been considered potentially dangerous, especially when performed by less-experienced endoscopists. Most authorities recommend that only experts perform a precut. However, recent studies have shown that the complications of precut sphincterotomy are similar to those associated with conventional sphincterotomy, namely bleeding, PEP, perforation, and cholangitis[33,43,46-49]. In terms of the endoscopist learning curve ensuring the safety and success of precut sphincterotomy, Akaraviputh *et al*[47] reported that the rate of procedure-related complications decreased significantly after the first 100 procedures were performed. Also, among all complications, the rate of immediate bleeding varied significantly, but the success of cannulation or the rate of PEP development did not differ with endoscopist experience. Lee *et al*[33] obtained similar results. The frequency of PEP in 159 patients who underwent precut fistulotomy did not differ by time interval. In the cited study, the risks associated with use of precut fistulotomy under circumstances where biliary cannulation was employed were not influenced by experience. Thus, the overall complication and PEP rates were similar; *i.e.*, not differing significantly, from those reported previously, at 10.7% and 5.7%, respectively, and the overall success rates were also similar, at 93.7%. No other serious complications were noted.

Consequently, most criticisms of the (supposedly) higher complication rates associated with precut sphincterotomy may be unwarranted. The high frequencies of post-procedural complications after such sphincterotomy may be associated with excessively edematous major papillae and extensive injuries caused by multiple or prolonged attempts to cannulate the CBD by standard methods before precut sphincterotomy. Huibregtse *et al*[49] showed that early implementation of precut increased successful biliary access on the first attempt, as well as the overall success rate, while reducing the rate of complications to 11.8% (pancreatitis: 0.5%). Previous repeat cannulation attempts, prolonged cannulation time, or numerous insertions of a guidewire into the pancreatic duct, may increase the risk of PEP. Freeman *et al*[3] reported that moderate numbers of cannulation trials (6-15), or more-than-moderate numbers (> 15), and use of more than one pancreatic contrast injection, were important in terms of the development of pancreatitis; multivariate analysis was used to arrive at these conclusions. Lee *et al*[33] showed that more than 15 attempts at cannulating the major papilla prior to precut fistulotomy was a risk factor for PEP development upon multivariate analysis (OR = 4.8, 95%CI: 1.178-19.580, *P* = 0.029). Bailey *et al*[41] also found that the number of attempts

at cannulating the papilla played a key role in guiding decision-making to minimize the risk of PEP. Thus, if precut fistulotomy is indeed a treatment candidate, early implementation of this approach may aid in successful selective biliary cannulation as well as reducing the severity of PEP. On the contrary, Cennamo *et al*[50] reported that the timing of precutting did not influence the operative success rate or the rate of complications associated with ERCPs. The cited authors showed that the rates of PEP did not differ between subgroups treated with early precutting (no more than 5 min of attempts at biliary cannulation using the standard approach, and three cannulations of the pancreatic duct) and delayed precutting (cannulation attempts lasting 25 min). However, the cited study had a small sample size and, thus, a low statistical power. A recent meta-analysis of early precut studies (although including precuts performed at different times and the use of various techniques including needle-knife precutting starting at the orifice, and fistulotomy) showed that early precut implementation reduced the PEP risk (to 2.5% *vs* 5.3%, OR = 0.47, 95%CI: 0.24-0.91) but not the overall complication rate[51].

Theoretically, the greater number of complications could have resulted from direct thermal injury caused by the needle-knife *per se*, especially during precut sphincterotomy, in which incisions commenced at the papillary orifice. Avoidance of thermal injury to the pancreatic duct, by making incisions above the papillary orifice during precut fistulotomy, minimizes the risk of pancreatitis[35,36,46]. However, too small a papilla, a short papillary roof, distortion caused by invasion of a tumor or a mass, or location of the ampulla of Vater on the inner center or ridge of a huge periampullary diverticulum, may preclude use of precut fistulotomy[33].

In summary, although some aspects of the timing and optimal type of precut sphincterotomy remain controversial, as does the need for endoscopist experience, use of early precut fistulotomy in patients for whom cannulation is difficult may not exacerbate PEP to an extent greater than conventional methods. In instances of persisting papillary contact or prolonged cannulation time, early precut fistulotomy may minimize the severity of PEP by decreasing mechanical trauma. However, a definition of a "difficult" cannulation, and adequate training in precut sphincterotomy are required, as are data from more large-scale multicenter studies.

Transpancreatic sphincterotomy/septostomy

Transpancreatic sphincterotomy or septostomy is a technique involving cutting of the septum that separates the pancreatic duct from the bile duct, through the pancreatic orifice[52]. Unlike a freehand technique such as use of a needle-knife, transpancreatic papillary septostomy in patients for whom cannulation is difficult, or who experience unintentional pancreatic duct cannulation, can be performed using a papillotome, without exchange of devices, after guidewire introduction into the pancreatic duct; or indeed without a guidewire. When unintentional

Table 2 Studies for the use of pancreatic stents to prevent post-endoscopic retrograde cholangiopancreatography pancreatitis

Study	Design	Indications	PEP rate		
			n	Non-stent/stent (%)	*P* value
Smithline *et al*[63]	RCT	Biliary ES for SOD, small ducts, or precut	93	18/14	0.229
Aizawa and Ueno[31]	Retrospective case-control	Biliary balloon dilatation for stone	40	6/0	0.110
Fogel *et al*[18]	Retrospective case-control	Biliary ± pancreatic ES for SOD	436	28.2/13.5	< 0.05
Fazel *et al*[32]	RCT	Difficult cannulation, biliary ES, SOD	76	28/5	< 0.05
Freeman *et al*[19]	Prospective case-control	Consecutive high-risk ERCP in which a major papilla PD stent was attempted	225	66.7/14.4	0.060
Harewood *et al*[58]	RCT	Endoscopic ampullectomy	19	33/0	0.020
Sofuni *et al*[64]	RCT	All consecutive ERCP (excluding pancreatic cancer, pancreas divisum, PD therapy cases)	201	13.6/3.2	0.020
Tsuchiya *et al*[66]	RCT	All consecutive ERCP irrespective of risk factors	64	12.5/3.1	> 0.05
Saad *et al*[70]	Retrospective nonrandomized	Suspected SOD and normal manometry	403	9/2.4	0.006
Lee *et al*[59]	RCT	Difficult biliary cannulation	101	29.4/12	0.031

ES: Endoscopic sphincterotomy; SOD: Sphincter of Oddi dysfunction; RCT: Randomize controlled study; PD: Pancreatic duct; PEP: Post-ERCP pancreatitis.

pancreatic duct cannulation has occurred, the procedure is relatively easy. Wire-guided septostomy is performed after introducing a soft guidewire into the pancreatic duct, and sphincterotomy follows, maintaining the bile duct orientation at 11 o'clock. If the septum between the pancreatic and bile ducts is incised, the biliary and pancreatic orifices become separately visible[52-54]. Another useful option for septostomy is a precut following placement of a prophylactic PS along the stent. This may primarily prevent PEP and also facilitates selective biliary access. This means that the second procedure, the precut from the orifice, is relatively easy; the operator is more comfortable in such circumstances than is the case when a freehand technique such as fistulotomy is to be performed. Either a precut from the orifice or fistulotomy is possible, but precutting from the orifice in the biliary direction along a supporting stent may be more feasible than use of the freehand technique. The prophylactic effects of PSs are described below.

PROPHYLACTIC PLACEMENT OF PANCREATIC STENTS

Pancreatic stents were originally introduced to treat pancreatic ductal pathology such as benign or malignant strictures and ductal leaks after trauma or surgery. The exact mechanism by which PSs may reduce the risk of PEP is but poorly understood. The stents probably preserve pancreatic drainage that otherwise might be impaired by mechanical injury to the pancreatic sphincter caused by prolonged or repeated manipulations of catheters and guidewires and thermal injury caused by biliary and pancreatic sphincterotomy or snare papillectomy. Many clinical trials and a meta-analysis have shown that placement of PSs in high-risk patients effectively reduces the incidence and/or severity of PEP. Recent studies have found that prophylactic placement of a PS reduces the frequency and severity of PEP in particular high-risk groups, including those with known or suspected SOD; those who have undergone papillectomy, precut

sphincterotomy, or pancreatic sphincterotomy; those with a history of PEP; or those for whom cannulation is difficult (Table 2)[18,31,32,55-70]. Prophylactic placement of PSs is now increasingly adopted to reduce the risk of PEP. PS placement also reduces the frequency of severe PEP[18-21,31,32,55-68].

Presently, the routine use of PSs in high-risk patients and in procedures conducted at advanced centers has changed attitudes toward ERCP; the incidence and severity of PEP have been reduced to more acceptable levels. However, few data are available on the effects of prophylactic PSs, especially in terms of technical difficulties in the context of cannulation time or the frequency of papillary contact[56,61,69]. Also, the sizes and lengths of the stents employed have been variable, and no guideline or consensus yet exists on the optimal type, diameter, or length of a PS.

Ideal types of pancreatic stents
PSs vary in terms of diameter, length, and shape. An ideal PS should completely prevent development of PEP, be easily deployed, spontaneously dislodge after exerting an adequate preventative effect, and not cause ductal or parenchymal pancreatic changes[71]. In terms of such changes, a retrospective analysis of 34 patients with 38 PSs placed to deal with disrupted ducts, isolated strictures, pancreas divisum, and hypertensive pancreatic sphincters, found that 36% of all patients exhibited subsequent ductal changes[72]. Also, a study on the dog pancreas showed that polyethylene PSs caused histopathological changes in normal tissues attributable to stent occlusion or local stent-induced trauma[73]. These results suggest that PSs may cause permanent changes to the pancreatic duct or parenchyme. If the placement time is too short, a smaller and shorter stent may not sufficiently protect against PEP development. Short stents (less than 3 cm long) are generally preferred to longer stents to avoid stenting across the neck of the pancreatic duct. However, longer stents should be considered when the pancreatic duct is angulated in the head of the pancreas. Stents may be straight, or may have a single pigtail or partial curl in the

Table 3 Efficacy of 3- *vs* 5-F pancreatic stents in preventing post-endoscopic retrograde cholangiopancreatography pancreatitis

	Technical success	Spontaneous migration	PEP	Stents
Rashdan *et al*[61] (3 F *vs* 4, 5, 6 F)	NA	86%/73%/67%/65%[1] (*P* < 0.01)	7.5%/10.6%/9.8%/14.6% (*P* = 0.047)	COOK, 4-12 cm
Chahal *et al*[56] (3 F *vs* 5 F)	91%/100% (*P* = 0.0003)	88%/98% (*P* = 0.0001)[2]	14%/9% (*P* = 0.3)	3 F, 8 and 10 cm/5 F, 3 cm
Zolotarevsky *et al*[69] (3 F *vs* 5 F)	97.5%/100%	75%/68.4% (*P* = 0.617)[2]	17.5%/10.5% (*P* = 0.519)	COOK, Zimmon 3 F, 3 cm/ 5 F, 5 cm

[1]10-14 d; [2]2 wk. PEP: Post-ERCP pancreatitis; NA: Not available.

duodenum, to prevent proximal migration. Short stents without proximal flaps facilitate early spontaneous migration (within 1 wk). Thus, establishment of drainage may be not assured when stents without proximal flaps are used because of the potential for very early stent migration. However, stents with flaps require endoscopic removal at a later date. Another option is to place longer (> 7 cm) stents of small diameter (3 or 4 F) that have no proximal flaps. This practice has the potential advantages of less ductal trauma and spontaneous distal migration; repeat endoscopy is not necessary[74]. A large retrospective study suggested that unflanged longer-length (8-10 cm) 3 F polyethylene stents with single duodenal pigtails were associated with significantly higher spontaneous dislodgement rates compared to larger-caliber, shorter unflanged 4 or 5 F stents. The cited study also reported a somewhat lower incidence of PEP in patients who received 3 F compared with 5 F stents, although the difference was not statistically significant[61]. Another study by Chahal *et al*[56] compared use of long 3 F and short 5 F stents and showed that the spontaneous dislodgement rate of unflanged, short 5 F PSs (98%) was significantly higher than that of unflanged, long 3 F stents (88%) after 14 days in patients at high risk for PEP development (*P* < 0.01). Placement of short stents reduced the need for later endoscopic stent removal. Higher rates of PS placement failure (0% in the 5 F group but 8.3% in the 3 F group, *P* = 0.0003) and PEP (14% in the 3 F group and 9% in the 5 F group, *P* = 0.3) were observed in patients with 3 F stents. Recently, Zolotarevsky *et al*[69] reported that placement of 5 F compared to 3 F PSs for PEP prophylaxis was easier, more rapid, and required fewer wires. However, no statistically significant differences in spontaneous passage rates (68.4% in the 5 F group; 75.0% in the 3 F group; *P* = 0.617) or PEP rates (*P* = 0.519) were evident (Table 3).

The prophylactic utility of placing smaller 3 F stents during difficult biliary cannulations has undergone little evaluation. Technically, the failure rates in previous studies involving placement of 3 F PSs after therapeutic ERCP have been rather high (9%-10%)[56,63]. The main problem is that a guidewire of smaller diameter than the standard 0.035-inch wire must be used. Deployment of long 3 F PSs is technically more difficult because of the need to use smaller caliber (0.018- or 0.021-inch) guidewires, which can be difficult to maneuver around tortuous pancreatic ducts compared to a hydrophilically tipped 0.035-inch guidewire. Placement of long stents also re-

quires deeper guidewire access into the main pancreatic duct, which may not be possible in patients with highly angulated or tortuous ducts. Thus, usually, placement of a 5 F PS using a 0.035-inch guidewire may be valuable to allow easy negotiation of the pancreatic duct and stent deployment. However, one recent randomized controlled trial evaluating the feasibility and utility of smaller and shorter (4-8 cm) 3 F stents showed that placement of a 3 F PS was technically feasible, significantly reduced the rate of PEP developing after difficult biliary cannulations, and that a higher rate of distal spontaneous dislodgement (94%) was evident within 7 d. The technical failure rate when experts operated was low (4%), and no complications resulted from PS placement[59]. The use of smaller-sized guidewires may require extensive endoscopic experience and skilled assistance.

Timing of pancreatic stent placement

It is unclear whether stents should be placed before or after therapeutic procedures such as sphincterotomy, stone extraction, and biliary stent placement, but early placement of a PS may be beneficial because various procedure-related factors may contribute to development of PEP. A retrospective study by Fogel *et al*[18] found that pancreaticobiliary sphincterotomy with PS placement was associated with a lower rate of pancreatitis than was biliary sphincterotomy alone. The cited authors noted a tendency for pancreatitis rates to be lower when a PS was placed before major papillar pancreatic or biliary sphincterotomy (10.7%), than after sphincterotomy (19.2%). Another retrospective study reported similar complication rates upon traction minor papillotomy followed by PS placement, compared with needle-knife surgery after PS placement (8.3% *vs* 7.8%)[75]. A recent randomized trial comparing use of the needle knife and pull-sphincterotome techniques for pancreatic sphincterotomy in high-risk patients showed that PEP was significantly more frequent among patients undergoing pancreatic sphincterotomy with a pull sphincterotome followed by placement of a PS than in those treated with needle-knife pancreatic sphincterotomy performed after placement of a PS [7 of 24 (29%) *vs* 0 of 24 (0%), *P* < 0.01]. Forty patients undergoing major papillar pancreatic sphincterotomy for manometrically documented SOD were randomized to traction sphincterotomy using a blended current followed by placement of a PS *vs* needle-knife sphincterotomy after placement of a stent; all patients received long, unflanged 3 F stents[76].

Access to the pancreatic duct after biliary sphincterotomy or other biliary therapy such as balloon dilatation or stone extraction is sometimes very difficult. Failure usually occurs either because the pancreatic orifice cannot be identified or a guidewire cannot be deeply advanced into the pancreatic duct. Also, deep pancreatic cannulation can be difficult or impossible when, anatomically, looping or tight angulations are evident in the distal pancreatic duct. For such reasons, it is recommended to access the pancreatic duct with a guidewire early in the procedure and to maintain wire access until a stent has been placed in high-risk cases in which PS placement is believed to be warranted[19,74]. However, sometimes, repeat procedures such as stone extraction using a retrieval balloon, or mechanical lithotripsy, may dislodge the guidewire or preloaded stent even though the stent was placed using a guidewire.

Usually, prophylactic PS placement, rather than only maintaining a guidewire, may be reasonable before any therapeutic procedure. This suggestion is based on the data of the studies reported above, but further large-scale, prospective studies are warranted.

Duration of pancreatic stent placement

Few data are available to indicate the duration for which a PS should remain in place to effectively prevent PEP. Cha *et al*[55] reported that the rates of pancreatitis were significantly higher in patients from whom PSs were removed immediately after needle-knife precut sphincterotomy compared to those in whom the stents were left in place for 7-10 d (21.3% *vs* 4.3%, *P* = 0.027). These data suggest that placement and maintenance of a PS when needle-knife precut sphincterotomy is performed reduces the frequency and severity of PEP. The cited study also showed that excessively early removal of a PS might not effectively prevent development of pancreatitis. However, no data regarding the adequacy of the duration of stenting that is needed to consistently prevent PEP are available. This may be anywhere from a few hours to a week or more. The precise duration of PS placement required to effectively reduce the risk of PEP is not well known.

In general, stent removal at the end of ERCP is not recommended. Excessively early removal of a PS may increase the risk of pancreatitis. However, removing a stent too late may increase the risk of ductal or parenchymal change. Ideally, the PS should be in place for a minimum of 24 h or more, and then dislodge spontaneously[2].

Pancreatic stent-related complications

Relapsing acute and chronic (painful) pancreatitis can develop in patients with pancreatic stent-induced injuries. However, the long-term outcomes of PS placement have not yet been thoroughly investigated although it is assumed that most ductal injuries are transient, eventually resolving spontaneously, without clinical symptoms. A large-scale retrospective study suggested that unflanged longer (8- to 10-cm), 3 F polyethylene stents with single duodenal pigtails were associated with a substantially reduced frequency of ductal change (24% for 3 and 4 F stents compared to 80% for 5 and 6 F polyethylene stents)[61]. This may indicate that use of smaller-caliber stents is associated with a reduced risk of ductal injury. Ductal and parenchymal changes may be most prominent in patients with traditional 5 or 7 F stents, because which may be of similar caliber to the native main pancreatic duct. The stent diameter should be less than that of the pancreatic duct.

Summary of use of prophylactic pancreatic stents

In prophylactic PS placement, a long PS of smaller diameter (3 F) may dislodge spontaneously within a few weeks without any ductal change, but small guidewires (0.018 or 0.021 inch) are required, and such small guidewires may be difficult to handle and to insert deeply into the tail portion. On the other hand, placement of short (2-3 cm) 5 F unflanged stents can commonly be achieved using 0.035-inch guidewires that can be handled relatively easily by endoscopists. Also, over 90% of such stents dislodge spontaneously. However, a stent that is too short may migrate soon after insertion, thus failing to prevent PEP and (perhaps) causing injury to the duct genu because of the short length. To effectively prevent PEP, the duration of stent placement that is adequate, without causing ductal or parenchymal change, should be determined. Finally, careful study of an ideal stent design, and the material used, is warranted. All of easy stent insertion, risk reduction, and spontaneous dislodgement in a timely fashion without ductal injury, are required. We suggest that short 5 F, or long 3 F, stents without inner flanges should be used to stent a normal pancreatic duct. The stent diameter should be less than that of the targeted pancreatic duct. However, endoscopists should remember that technical failure of PS insertion might aggravate the severity of pancreatitis, so that the procedure *per se* unfortunately becomes a risk factor.

OTHER ALTERNATIVES

Repeat or delayed ERCP

Repeat or continuing attempts at cannulation increase the risk of PEP, as explained above. When primary cannulation fails, the alternatives include PTBD, repeat ERCP conducted by same or another endoscopist (perhaps in a more advanced institution) after 2-3 d of delay, or surgical exploration[77,78]. Of these approaches, delayed ERCP performed 2-3 d later by the same endoscopist may increase the success rate of selective cannulation and also reduce the complication risk to within an acceptable range by avoiding excessive papillary manipulation or unintentional ductal injury. Delayed ERCP may afford a good visual field, without papillary edema or bleeding, and reduce the rapid bowel movement that develops with longer procedure times, in turn reducing the need for additional procedures and enhancing successful biliary cannulation. However, excessively prolonged manipulations during primary cannulation attempts are inevitably

associated with complications. A decision to interrupt a procedure should be considered as early as possible.

Percutaneous transhepatic biliary drainage

Percutaneous transhepatic biliary drainage (PTBD) is the most common salvage procedure used to access the biliary tract after failure of ERCP. Especially in patients with advanced malignant hilar biliary strictures, percutaneous drainage may be more feasible than endoscopic drainage. To palliate jaundice in patients with non-resectable malignant hilar biliary strictures, the biliary obstruction pattern (particularly the Bismuth type) should be considered before selection of an optimal drainage method. Endoscopic biliary drainage and stenting is recommended as the first-line drainage procedure in Bismuth type II patients, considering that this approach is efficacious and relatively noninvasive. However, internal stent insertion and drainage through the PTBD tract may be the best option for Bismuth type IV[79] patients. One retrospective study found that the success of biliary drainage was significantly higher when drainage was percutaneous rather than endoscopic (93% *vs* 77%, *P* = 0.049)[80]; no between-group differences in overall complication rates or the median survival time of successfully drained patients were evident. The goal of palliative drainage of hilar cholangiocarcinoma patients is drainage of an adequate liver volume (50% or more), irrespective of unilateral, bilateral, or multisegmental stenting. In patients of Bismuth types III or IV, the percutaneous approach was preferred over the endoscopic approach in a document detailing Asia-Pacific consensus recommendations[81]. However, PTBD-related adverse event rates of 9%-33%, and mortality rates of 2%-15%, have been reported[82-85]. Furthermore, in terms of quality of life, long-term placement of external catheters is very uncomfortable for patients. Also, recent studies on bilateral metallic stenting have enjoyed high levels of technical success, and a reduced revision rate, even in patients with advanced hilar cholangiocarcinoma[86-88].

The choice of an endoscopic approach or PTBD may depend on endoscopist experience and institutional guidelines. In the near future, advanced endoscopic techniques and newly developed devices may improve endoscopic methods. However, in terms of complications, in particular PEP, primary PTBD does not irritate the ampulla of Vater. Accordingly, in difficult cases, and in advanced hilar cholangiocarcinoma patients requiring adequate drainage, PTBD can be both an alternative option and a rescue method.

FUTURE ADVANCES IN ENDOSCOPIC PROCEDURES

Repeated cannulation attempts and pancreatic duct manipulations on the ampulla of Vater are associated with PEP development, caused by inevitable contact with the papilla. Thus, theoretically, PTBD, or EUS-guided biliary drainage (EUS-BD), may serve as alternatives to ERCP

when it is performed to inhibit development of pancreatitis by avoiding direct contact with the ampulla of Vater. Recent studies have shown that EUS-BD is an effective alternative to PTBD after failure of ERCP. Also, a potential benefit of EUS-BD is internal drainage, thereby avoiding long-term external drainage in patients who are expected to enjoy longer survival and in those for whom external PTBD drainage catheters cannot be internalized. However, EUS-BD with transluminal stenting is inherently complex in procedural terms, requiring several multi-step processes, thus prolonging procedure times, in turn associated with the possible development of several adverse events, including stent migration and bile peritonitis[89-93]. Also, EUS-guided drainage techniques have been but recently developed and no dedicated devices or guidelines are yet available. Procedure-related complications including bile peritonitis or pneumoperitoneum are not uncommon. To date, the procedure has been performed by only experienced endoscopists in advanced endoscopy centers, usually as a salvage method rather than as a form of primary biliary drainage. Further development of technical devices and establishment of standard techniques minimizing complications are needed. Also, further long-term follow-up in the context of large-scale studies (including primary intervention to ensure biliary drainage) are required before the technique can be recommended for primary use.

SUGGESTED ALGORITHM FOR ENDOSCOPIC PREVENTION OF POST-ERCP PANCREATITIS

Prior to ERCP, patient selection considering risk stratification, operator-related factors, and hospital circumstances, should be considered, and efforts should be made to avoid unnecessary ERCP by diagnostic replacement with EUS or MRCP, if possible. Trainee involvement must be taken into account. If possible, pharmacological prophylaxis - such as rectal NSAIDs - should also be considered. We recommend wire-guided rather than conventional cannulation as the initial cannulation method. If unintentional pancreatic duct cannulation occurs more than three times, it may be wise to consider changing to double-guidewire cannulation or transpancreatic septostomy to enhance biliary access. However, in such instances, precutting from the orifice following early prophylactic PS placement may be more effective to reduce the severity of PEP. If attempts at double-guidewire cannulation persist for some time or a technical difficulty is encountered, an early switch to a precut following prophylactic PS placement should be considered. Also, the use of a double-guidewire cannulation technique may increase the risk of complications caused by additional frequent papillary contact, or pancreatic duct cannulation, even though use of the method may facilitate selective biliary cannulation. Transpancreatic sphincterotomy may be also a risk factor for PEP if pancreatic juice passage is disturbed.
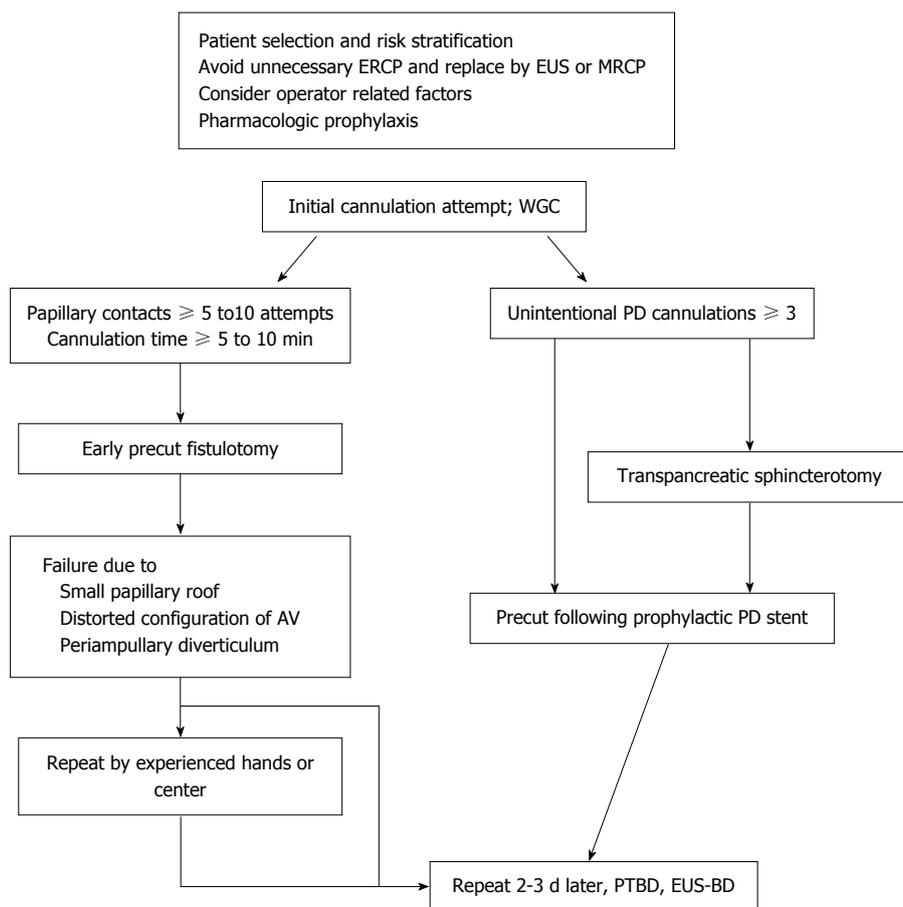
Figure 1 Suggested endoscopic algorithm for decreasing the severity of post-endoscopic retrograde cholangiopancreatography pancreatitis and facilitating biliary access. WGC: Wire-guided cannulation; AV: Ampulla of Vater; PD: Pancreatic duct; PTBD: Percutaneous transhepatic biliary drainage; EUS-BD: EUS-guided biliary drainage.

Thus, risky operative conditions, such as a prolonged procedure time (more than 5-10 min), or technical failure of selective cannulation, should trigger consideration of prophylactic pancreatic stenting. Otherwise, if frequent papillary contacts persist (if more than 10 cannulation attempts, or at the very most up to 15 attempts, are made), or the cannulation time is more than 5-to-10 min without unintentional pancreatic cannulation, early precut fistulotomy can be considered. However, if the papilla is too small, the segment of the papillary roof short, a periampullary diverticulum present, or the ampulla is located in the center of the ridge of the diverticulum, a precut may be disturbed. In those cases, PTBD, EUS-BD, the rendezvous technique, repeat ERCP performed by a senior experienced endoscopist, or delay in ERCP for 2 or 3 d, should be considered. Use of such a step-wise algorithm may enhance successful biliary access and avoid unnecessary prolongation of procedure time (Figure 1). However, such options should be considered against a background of hospital circumstances and the availability of endoscopists.

CONCLUSION

Various endoscopic or interventional techniques includ-

ing primary wire-guided cannulation, precut fistulotomy, transpancreatic septostomy, prophylactic PS placement, or alternatives such as PTBD or EUS-BD, have been described above as prophylactic methods for the decreasing severity or frequency of PEP. Till now, prophylactic PS placement in high-risk patients or those treated with certain procedures may be the single most effective method to reduce the severity and/or frequency of PEP. Improvements in stent design and the materials used in stent construction are to be expected. Also, the optimal timing of stent placement and its duration require study. Wire-guided cannulation and precut fistulotomy should be compared using strict definitions of "difficult" cannulation, endoscopist experience, and racial or regional characteristics. Furthermore, as either alternative or primary methods, PTBD or more advanced EUS-guided techniques may be available in difficult or failed cannulation. Finally, recently emerging pharmacological prophylaxis, such as rectal NSAIDs, should be considered either in combination, or alone, in large-scale comparative studies.

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Pharmacologic therapy for acute pancreatitis

Swetha Kambhampati, Walter Park, Aida Habtezion

Swetha Kambhampati, Walter Park, Aida Habtezion, Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, United States
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Correspondence to: Dr. Aida Habtezion, MD, Division of Gastroenterology and Hepatology, Department of Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305, United States. aidah@stanford.edu
Telephone: +1-650-7253362 Fax: +1-650-7235488
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Abstract

While conservative management such as fluid, bowel rest, and antibiotics is the mainstay of current acute pancreatitis management, there is a lot of promise in pharmacologic therapies that target various aspects of the pathogenesis of pancreatitis. Extensive review of preclinical studies, which include assessment of therapies such as anti-secretory agents, protease inhibitors, anti-inflammatory agents, and anti-oxidants are discussed. Many of these studies have shown therapeutic benefit and improved survival in experimental models. Based on available preclinical studies, we discuss potential novel targeted pharmacologic approaches that may offer promise in the treatment of acute pancreatitis. To date a variety of clinical studies have assessed the translational potential of animal model effective experimental therapies and have shown either failure or mixed results in human studies. Despite these discouraging clinical studies, there is a great clinical need and there exist several preclinical effective therapies that await investigation in patients. Better understanding of acute pancreatitis pathophysiology and lessons learned from past clinical studies are likely to offer a great foundation upon which to expand future therapies in acute pancreatitis.

Core tip: Currently there are no approved therapies for acute pancreatitis. This review summarizes and discusses pre-clinical and clinical studies in acute pancreatitis and also discusses potential promising therapies.

INTRODUCTION

In the United States in 2009, over 274000 patients were diagnosed with acute pancreatitis making it the most common gastrointestinal disease requiring acute hospitalization with costs for treatment exceeding $2.5 billion annually[1]. The prognosis of patients with acute pancreatitis is largely determined by the presence of organ failure and infected pancreatic necrosis with associated mortality rates of 15%-30%[2]. Other complications of pancreatitis include systemic inflammatory response syndrome (SIRS), sepsis, and acute respiratory distress syndrome (ARDS). Despite the increasing incidence[3], there is no current available pharmacologic therapy to mitigate the disease and its course. Current treatment of pancreatitis is largely supportive. Treatment of organ failure consists of organ supportive measures[4], while treatment of infected pancreatic necrosis consists of drainage or

debridement and antibiotics[5].

The most common causes of acute pancreatitis in the Western population are alcohol and gallstones, but many other causes have also been described and regardless of the trigger, there is an underlying common pathogenic outcome[6,7]. Acute pancreatitis is thought to be a local inflammatory process involving premature intra-cellular activation of digestive enzymes within acinar cells leading to autodigestion of the tissue that can progress to involve distant organs. The secretory acinar cells are also thought to release chemokines and cytokines that recruit leukocytes triggering an inflammatory response responsible for pancreatic edema and neutrophil accumulation[8]. This interstitial edema can sometimes progress to necrosis in parts of the pancreas and possible bacterial infection. Acute pancreatitis can also affect the microvascular circulation and compromise perfusion and oxygenation of the tissue[9]. While supportive therapy is largely the only treatment available for this disease, research in pancreatitis and a better understanding of the pathophysiology has led to the development of some pharmacologic therapies that target the various steps in the pathogenesis of pancreatitis. A review published in 2008 outlined some of the pharmacologic therapies investigated in experimental animal models[10]. Since then, some of those therapies have been further studied, new drugs have been found in each class of therapy, and more human clinical studies assessing the clinical utility of the therapies have been conducted.

This review focuses on the newer pharmacologic therapies for treatment of acute pancreatitis, and does not address the pharmacology of the standard treatment currently used such as pain control and antibiotics. The article summarizes the experimental, pre-clinical studies that provide evidence for the therapeutic potential for various classes of newer medications, outlines the clinical trials that have assessed their translational potential, and comments on future therapies and potential promising agents awaiting translation to clinical practice. This review focuses on pharmacologic therapy for acute pancreatitis that is not secondary to endoscopic retrograde cholangio-pancreatography (ERCP).

MOLECULAR PATHOGENESIS OF PANCREATITIS

Under physiologic conditions, inactive enzyme precursor secretion from the acinar cell occurs in response to cytosolic calcium. A sustained global elevation of this calcium, however, can lead to premature activation and secretion of digestive enzymes from the acinar cell, one of the earliest detectable events in pancreatitis[11]. After the initial insult to the pancreatic acinar cell, the disease progression is a multi-phase process that involves local inflammation of the pancreas, followed by a generalized inflammatory response, and the final stage of sepsis involving multi-organ failure in those with severe disease[8]. Following the initial injury, inflammatory cells are

often recruited to the pancreas *via* adhesion molecules, which can aggravate the inflammatory response leading to severe acute pancreatitis[8]. One of the key drivers of the inflammatory response in acute pancreatitis is likely circulating cytokines and chemokines. Active digestive enzymes are potent stimulators of macrophages, which subsequently induce the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins[12].

Cytokine production is governed by a large number of transcription factors, most prominent of which is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[12]. The various types of cytokines released can cause their effects *via* highly specific cell surface receptors and stimulate enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase (iNOS), which mediate the inflammatory process. Hence inhibition of these enzymes is likely to limit the local and systemic injury induced by pro-inflammatory leukocytes[12]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have also been implicated in the pathogenesis of acute pancreatitis. The mechanism by which these agents induce pancreatitis is two-fold. ROS and RNS act directly on biomolecules (lipids, proteins, and nucleic acids) and oxidize these components of cell membrane in the pancreas leading to membrane disintegration and necrosis of the pancreatic cells. In addition to the direct detrimental oxidative effects, ROS and RNS can also serve as secondary messengers in intracellular signaling and induce pro-inflammatory cascades[13].

PRECLINICAL STUDIES

Anti-secretory agents

Acute pancreatitis is characterized by pancreatic and peripancreatic fat injury in part mediated by autodigestive enzymes. Excessive stimulation of the exocrine pancreas worsens acute pancreatitis[9] and thus is the rationale for testing anti-secretory agents as potential therapies for acute pancreatitis. Initial animal studies in the 1970s tested glucagon and subsequent studies investigated the use of somatostatin and long-acting somatostatin analogue.

Glucagon increases superior mesenteric artery blood flow and decreases pancreatic exocrine secretion[14]. A study utilizing a dog model of pancreatitis, however, did not find glucagon treatment alone or in combination with volume resuscitation to be better than volume resuscitation alone[15]. In fact in their model, pancreatic hemorrhage was associated with glucagon treatment suggesting possible worsening of the disease. A later study using pigs reported beneficial effects of glucagon[16] but other experimental studies in addition to the study mentioned above failed to support the use of glucagon therapy in experimental acute pancreatitis[17-19].

Somatostatin is an inhibitory hormone with multiple effects on gastrointestinal motility and exocrine pancreas secretions[20]. One preclinical study using a taurocholate-induced rat model of acute pancreatitis, showed that so-

matostatin was effective in inhibiting basal and hormonal stimulated pancreatic enzyme secretion but did not affect the degree of pancreatic necrosis, pancreatic edema, leukocyte infiltration, or the enzyme content of the pancreas after pancreatitis was induced and did not lead to an overall decrease in mortality[21]. Another study showed that somatostatin stimulates hepatic and splenic reticulo-endothelial function in the rat hence suggesting benefit in the treatment of pancreatitis[22]. Preclinical studies have showed benefit of using somatostatin and its long-acting analogue, which provides the basis for the clinical trials discussed below.

The utility of anti-secretory agents has limitations given that the pancreas not only secretes enzymes, but also secretes bicarbonate and fluids, and animal studies have shown that stimulation of ductal secretion of bicarbonate has a protective effect on the severity of pancreatitis[23].

Protease inhibitors

Intrapancreatic activation of digestive enzymes plays an important role in the pathogenesis of acute pancreatitis. For this obvious reason protease inhibitors have been and remain of therapeutic interest in acute pancreatitis. Early studies in dogs with surgically-induced pancreatitis treated with trypsin inhibitors from egg white or soybean, and trasylol (aprotinin), a trypsin-kallikrein inhibitor from cattle were effective in suppressing acute pancreatitis[24]. Several other animal studies, including guinea pig model with taurocholate-induced necrotizing pancreatitis, also showed benefit with using protease inhibitors such as chlorophyll-a[25,26]. Interestingly however in the choline-deficient DL-ethionine (CDE) supplemented diet model of severe hemorrhagic pancreatitis, neither trasylol nor chlorophyll-a resulted in disease or mortality attenuation[27]. Despite the use of protease inhibitors at the time of CDE acute pancreatitis induction, the difference in rapidity, extent and intracellular protease release as well as the degree of and/or drug tissue penetration associated with the different experimental models might contribute to the observed opposing results[23]. Nevertheless, the later study has been more consistent with the clinical trial findings discussed below.

Anti-inflammatory and immunomodulators

Altered products of arachidonic acid metabolism have been detected in experimental acute pancreatitis[28]. In acute pancreatitis thromboxane B levels are elevated whereas levels of prostaglandin E (PGE) are decreased. PGE therapy has been shown to have protective effects on the course of experimental acute pancreatitis in rodent models with taurocholate, CDE diet, or caerulein-induced pancreatitis[29-31]. Cyclo-oxygenase inhibitors such as indomethacin have been used to treat experimental acute pancreatitis with conflicting results in earlier studies[32] but subsequent studies have supported the beneficial effect of indomethacin particularly when used early or prior to disease induction in rat models with tauro-cholate-induced acute pancreatitis[33,34]. Similar to cyclo-

oxygenase, lipoxygenase is downstream of arachidonic acid and studies have shown that its inhibition in rat models with taurocholate-induced acute pancreatitis leads to reduced severity of the disease[35]. Leukotriene receptor antagonism however has shown to either worsen or have no effect on rat acute pancreatitis[33,36], indicating the complexity of the pathway and the need for further in depth investigations.

Steroid therapy in acute pancreatitis has been of interest for several decades due to the associated leukocyte activation and release of inflammatory cytokines and chemokines during progression of acute pancreatitis. One experimental study in rat acute pancreatitis caerulein-induced showed that the effectiveness of steroid therapy depended on the severity of illness, with dexamethasone being more effective against pancreatitis with severe inflammation[37]. Another experimental study showed that dexamethasone reduced pancreatic damage when given prophylactically through reduction in intercellular adhesion molecule-1 expression, but was ineffective in preventing leukocyte recruitment into the pancreas when given therapeutically to rats with taurocholate-induced acute pancreatitis[38]. On the other hand, steroids have also been implicated as a cause of acute pancreatitis and some studies show that high-dose hydrocortisone can increase mortality rate and complication rates such as sepsis or infection[39-43].

Animal studies have shown that interleukin-10 (IL-10), an anti-inflammatory cytokine, can ameliorate the severity of caerulein-induced acute pancreatitis in rodent models if given before or after the induction of disease[44]. IL-10 plays a protective role in the local and systemic consequences of the disease as IL-10 has been shown to block inflammation leading to improved outcome in experimental models[45]. Lexipafant, an antagonist of the platelet activating factor[46], induces systemic effects and has been implicated as a mediator in the progression of acute pancreatitis[47]. Studies have shown that treatment of taurodeoxycholate and caerulein-induced rodent models of pancreatitis with lexipafant reduces the severity of pancreatitis-associated complications such as intestinal dysfunction, systemic IL-1 upregulation, and local leukocyte recruitment[48]. Lexipafant reduced acute pancreatitis associated inflammation[49] and improved acute necrotizing pancreatitis[50]. Despite beneficial outcomes with IL-10 and lexipafant in animal studies, their translation into clinical use has proven to be challenging.

Anti-oxidants

Oxidative stress and injury is implicated in many inflammatory diseases. Oxygen-derived free radicals are generated in experimental acute pancreatitis[51] and there is evidence of decreased blood anti-oxidant levels in patients with severe acute pancreatitis[52]. These observations led to experimental studies that showed a protective effect of exogenously administered anti-oxidants such as selenium through reduction of pancreatic injury[53]. Pre-clinical studies in rodent models of acute pancreatitis induced by

a variety of methods, including carrageenan injection into pleural cavity or *L*-arginine hydrochloride, have shown reduced levels of glutathione and increased levels of oxidized glutathione suggesting a benefit from this intervention[54]. Clinical studies, which will be discussed later in detail, however have not been positive and it may be that anti-oxidants are more useful in prevention and/or as synergistic agents.

Potential future promising therapeutic targets

One of the major limitations of preclinical studies is the uncertainty and lack of an ideal model that recapitulates all aspects of human disease. In addition preclinical therapies are often given either early or at the time of acute pancreatitis induction, when in reality patients often present with ongoing or late onset acute pancreatitis. Preclinical studies that demonstrate the efficacy of the therapy when administering the drug following disease progression are more likely to yield promising outcomes in clinical trials. With the exception of ERCP-induced acute pancreatitis in which prophylactic therapies can be instituted, it is unlikely that agents that interfere with initiation of acute pancreatitis are going to be effective but rather those that target the subsequent injury, repair or inflammatory pathways are likely to be beneficial in treating acute pancreatitis. For this reason, we discuss a few of the promising preclinical studies that target these pathways.

Inducible nitric oxide synthase activity is thought to be increased in experimental acute pancreatitis[55]. Experimental studies in the rat model have shown that agents such as *S*-methylisothiourea, an inducible nitric oxide synthase inhibitor, can decrease the bacterial translocation from gut into pancreatic necrosis thus reducing septic complications and mortality in acute pancreatitis[56]. Treatment with agents such as AR-C102222AA or *L*-N^6-(1-iminoethyl)-lysine, highly selective iNOS inhibitors, early in the course after induction of acute pancreatitis, have also shown to have significant beneficial effects in acute pancreatitis in Australian possums[57] and may offer therapeutic benefit by decreasing pancreatic injury in future clinical studies.

Experimental studies with both pentoxifylline and heparin have shown a protective effect in rat models with acute pancreatitis. Studies have shown that pentoxifylline attenuates neutrophil activation, proinflammatory signalling, and systemic inflammation and cytokine levels in experimental acute pancreatitis especially when administered early[58]. Heparin has also been a pharmacologic therapy of recent interest with increasing number of reports suggesting its potential in the treatment of acute pancreatitis. Heparin was initially studied primarily for its ability to improve microcirculation, given that a disruption of the microcirculation contributes to the inflammatory process of acute pancreatitis. The anticoagulation mechanism of heparin is also associated with its anti-inflammatory effect in part secondary to reduced stimulation of macrophages and monocytes[59]. Experimental studies show that addition of heparin results in a decrease

of amylase, endothelin-1, and inflammatory cytokines such as TNFα, activation of NF-κB, and improved morphologic changes and vascular flow in the pancreas[60]. Such agents may enhance healing while dampening proinflammatory pathways, and may offer benefit in clinical acute pancreatitis.

Up-regulation of hemeoxygenase-1 (HO-1) or treatment with its downstream effectors and heme degradation products, biliverdin and carbon monoxide have protective effects in different rodent models of acute pancreatitis induced by taurocholate, caerulein, or CDE diet[61-66]. HO-1 overexpressing macrophages protect against acute pancreatitis[61,67]. Panhematin, an FDA-approved hemin formulation for acute intermittent porphyria, can prime HO-1 production[68]. Studies have shown that Panhematin if given before development of experimental pancreatitis can upregulate hemin-activated macrophages and lead to less pancreatic injury and if given after the development of acute pancreatitis, can also mitigate the extent of pancreatitis-related injury[63]. Notably, peripheral blood mononuclear cells from patients with mild acute pancreatitis have reversible HO-1 up-regulation that is associated with clinical recovery supporting therapeutic potential of HO-1 and the heme degradation products in patients with acute pancreatitis. Biliverdin *via* the aryl hydrocarbon receptor up-regulates IL-22[66]. There has been a lot of interest recently on the role of IL-22, a cytokine produced by hematopoietic cells that targets non-immune cells[66]. The pancreas interestingly has the highest expression of IL-22 receptor amongst any other tissue[69] and IL-22 treatment has been shown to ameliorate experimental acute pancreatitis[70]. Thus HO-1 and its downstream effectors are potential targets for clinical acute pancreatitis.

TNFα plays a central role in the pathogenesis of local and distant complications of acute pancreatitis[71], and its blockade ameliorates experimental acute pancreatitis induced by caerulein, taurocholate, or CDE diet in mice studies[72,73]. Although there are theoretical increased risk of infectious complications with anti-TNF therapy, there are case reports with positive outcome in acute pancreatitis patients who received anti-TNF due to concomitant medical illness for which the anti-TNF therapy was indicated[74,75]. Thus with careful patient selection it is likely that anti-TNF therapy will yield beneficial results in clinical trials.

CLINICAL STUDIES

Based on the pathophysiology of acute pancreatitis and the basic science research conducted providing evidence for promising pharmacologic therapy, many clinical studies have been performed assessing the effectiveness of these therapies including anti-secretory agents, protease inhibitors, immunomodulators, anti-inflammatory agents, and anti-oxidants.

Anti-secretory agents

The use of glucagon for acute pancreatitis was first re-

ported in 1971 by Knight *et al*[76] and since then several subsequent uncontrolled clinical trials have shown a clinical improvement, decrease in pain, and a decline in enzyme activities in acute pancreatitis[77-79]. However, in a subsequent double-blinded trial of 69 patients glucagon was not found to have a significant impact on the mortality of the patients when compared to placebo[80]. Further clinical trials found no difference in mortality and morbidity such as pain and length of stay[81,82].

Atropine was also studied in a randomized clinical trial but did not have a significant effect on the clinical course of patients when compared with no treatment[83]. Infusion of salmon calcitonin was also thought to strongly depress gastric secretions such as gastric acid, pepsin, and gastrin[84-86] as well as pancreatic enzyme secretions stimulated by various secretagogues[87] without affecting the fluid and electrolyte secretions[87] thus mitigating the pathogenesis of acute pancreatitis[88]. A multicentered randomized double-blinded trial assessing the use of synthetic salmon calcitonin in acute pancreatitis showed that though mortality was not affected, the number of patients without pain and normalized serum amylase was higher in the treated group as compared to the placebo group. Other parameters such as analgesic dose, leukocyte count, and normalization of seven clinical and laboratory criteria showed a positive trend in the treated group but was not clinically significant[88].

Clinical trials have well studied the use of somatostatin for treatment of acute pancreatitis given that it inhibits pancreatic exocrine secretions, reduces splanchnic blood flow, stimulates the hepatic reticuloendothelial system, and modulates the inflammatory cytokine cascade[89]. However, several randomized clinical trials failed to show a clinically significant benefit with the use of somatostatin[90-94]. A meta-analysis of seven publications, on the other hand, did show an overall mortality benefit with somatostatin for severe acute pancreatitis (OR = 0.36, 95%CI: 0.2-0.64) but there was no significant decrease in complication rates in patients with acute pancreatitis[95].

Octreotide, a synthetic analogue of somatostatin, was also tested clinically. While initial small studies did not show any overall mortality benefit, they suggested a decrease in severity of acute pancreatitis, reduced local complications, and earlier return to oral intake[96]. One of the largest clinical trials of 302 patients with moderate to severe pancreatitis however did not show any clinical benefit[97], but a smaller study of 50 patients with severe acute pancreatitis showed a clinically significant reduction in sepsis (76%-24%), ARDS (56%-28%), hospital stay (33.1-20.6 d), and mortality (8-2 deaths)[98]. While an older meta-analysis performed did suggest a mortality benefit for severe acute pancreatitis (OR = 0.57, 95%CI: 0.35-0.88)[95], another more recent meta-analysis that limited their estimate to four higher quality studies did not show any benefits in sepsis, mortality, or complication rates[99].

Thus clinical studies assessing the use of anti-secretory agents have provided inconclusive evidence on their ben-

efits. There appears to be no benefit with the use of these agents in mild acute pancreatitis, and the benefits are uncertain in severe acute pancreatitis. Hence these agents are not currently recommended in clinical practice[4,100].

Protease inhibitors

One of the earliest protease inhibitors studied is aprotinin, and initial studies showed some benefit in mortality though subsequent studies have failed to repeat such results[101,102]. Studies delivering aprotinin *via* peritoneal lavage have shown less necrosis in the treatment group with a reduction in complement activation (specifically less C3a and more C1 inhibitor plasma levels) but no overall difference in mortality[103-105]. Aprotinin may still have a role in treating acute pancreatitis given that it was not given in high enough doses to produce sufficient inhibition of protease activity and the studies were not adequately powered[106].

Gabexate mesilate is a smaller protease inhibitor that has been studied in human clinical trials[107]. While early smaller clinical studies suggested a mortality benefit with the administration of this therapy[108,109], a larger randomized controlled trial of patients with moderate to severe acute pancreatitis found no clinical benefit[110,111]. Two other meta-analyses also demonstrate this lack of mortality benefit, though they showed a decreased need for surgery and less complications[95,112]. A recent study showed some benefit in gabexate when delivered through continuous regional arterial infusion (CRAI)[113].

Nafomostat is a newer synthetic protease inhibitor a hundred times more potent than gabexate[107]. Clinical studies assessing the delivery of nafomostat *via* CRAI along with antibiotics have shown greater mortality benefit and lower incidence of necrosis with earlier administration of the drug[114]. Studies have also shown that delivery of the drug *via* CRAI compared to non-CRAI decreases the need for surgery and improves survival[115,116].

None of the protease inhibitors mentioned above are currently part of standard clinical care for acute pancreatitis treatment as larger and adequately powered studies are needed prior to their recommendation for clinical use. Nafomostat, however, has the most promise out of the three particularly when given *via* CRAI in combination with antibiotics.

Immunomodulators

Based on the preclinical positive results, Lexipafant was tested in clinical trials in patients with severe acute pancreatitis. The first clinical trial assessing the use of this therapy did not show a difference in mortality but showed a reduction in organ failure[117]. Another study showed significantly less organ failure, a reduction in mortality and SIRS[118]. The largest randomized clinical trial involving this therapy, however, showed no significant reduction in organ failure or local complications leading to the conclusion that lexipafant alone cannot treat severe acute pancreatitis[119].

Dotrecogin alfa, an analogue of endogenous protein

C, has shown some benefit in the treatment of acute pancreatitis[120]. Endogenous protein C is made in the liver and inhibits thrombin formation and facilitates thrombolysis. Given that lower levels of activated protein C are associated with higher mortality in acute pancreatitis, activated protein C was thought to mitigate severe acute pancreatitis by modulating the immune system through regulating leukocyte endothelial interaction and mitogen-activated kinases and improving intestinal microcirculation[121]. Initial case reports showed some benefit in using dotrecogin alfa in acute pancreatitis[122], but a subsequent pilot study did not show any clinically significant difference with the use of dotrecogin alfa[123].

Anti-inflammatory agents

Indomethacin, which inhibits phospholipase A2 activity and cyclooxygenase activity thus decreasing neutrophil mediated inflammation, has been clinically studied based on earlier pre-clinical studies[124]. One study assessing this therapy however, only reported decreased pain and opiate use when given to patients with acute pancreatitis[125] suggesting analgesia but not anti-inflammatory related benefits. So far benefits of indomethacin have been largely limited to post-ERCP pancreatitis[126].

Steroid therapy is widely used to dampen inflammation in various organ systems. Though steroid therapy has been shown to be beneficial in the treatment of auto-immune pancreatitis[127], in acute pancreatitis however steroid therapy has been implicated in disease induction[128]. A postmortem study done by Carone and Liebow showed histologic evidence of acute pancreatitis or peripancreatic fat necrosis in 16 out of 54 patients treated with steroids[129]. Initial case reports have also linked the use of steroids with acute pancreatitis[130]. Studies have also shown that corticosteroids have no beneficial effect in the prevention of post-ERCP pancreatitis[131]. However, given that some pre-clinical studies suggest that steroids can reduce the inflammatory cascade, leukocyte recruitment, and subsequent pancreatic damage when given prophylactically[38], further well-designed studies are warranted.

Anti-oxidant agents

Several clinical trials have assessed the benefit of anti-oxidant agents in acute pancreatitis given the role of reactive oxygen species and cellular injury in acute pancreatitis as well as the evidence generated by pre-clinical studies. Anti-oxidant agents studied include n-acetylcysteine, methionine, beta-carotene, selenium, ascorbic acid, and alpha-tocopherol.

A randomized clinical trial assessing treatment with acetylcysteine, selenium, and vitamin C showed increased serum levels of anti-oxidants and decreased markers of oxidative stress but no improvement in organ dysfunction[132]. Another study with patients receiving Vitamin C, n-acetylcysteine, and other anti-oxidants showed no significant difference in complications or length of hospital stay[133]. The third recent clinical study with vitamins A, C, and E also showed no significant difference in organ dysfunction[134].

Studies assessing the use of glutamine, a more potent anti-oxidant, have been more promising. One study randomizing 80 patients to glutamine showed decreased number of complications, length of stay, need for surgery, and mortality when administered early after hospitalization[135]. A meta-analysis of randomized control trials with glutamine showed a mortality benefit (RR = 0.3, 95%CI: 0.15-0.6) and reduced infectious complications (RR = 0.58, 95%CI: 0.39-0.87), but no difference in length of hospital stay. The benefit with glutamine was observed only in patients receiving total parenteral nutrition[136]. Thus the role of anti-oxidant therapy in acute pancreatitis remains to be determined.

Other therapies

A variety of other therapies for acute pancreatitis have also been assessed in clinical studies. Antifibrinolytics such as epsilon-aminocaproic acid (EPCA) has been thought to ameliorate the pathogenesis of acute pancreatitis by inhibiting the activation of plasminogen, plasmin, and trypsin, by inhibiting pancreatic kallikrein, and by increasing serum antitrypsin activity[137]. A clinical study assessing the use of EPCA and aprotinin in acute pancreatitis, however, did not have any clinically significant improvement on outcomes such as hospital duration and normalization of laboratory values compared to the conventional treatment group and the aprotinin treated groups[138].

Fresh frozen plasma (FFP) has also been assessed in the treatment of acute pancreatitis given laboratory studies that showed the inhibitory effect of FFP on proteolytic activity in the serum of patients with acute pancreatitis[139]. While one initial prospective pilot clinical study showed a decrease in mortality with the administration of FFP in patients with acute pancreatitis when administered during the first five days of illness onset[139], a larger multi-centered controlled clinical trial showed no improved clinical outcome in the group given FFP as opposed to colloids treated group[140].

Molecular pathways under target development include the kallikrein-kinin and complement system given that severe acute pancreatitis is associated with elevated C3a and sC5b-9 levels[141]. C1 esterase inhibitor blocks a variety of proteolytic enzymes including activated C1 complex and kallikrein[142], and both experimental studies as well as small human studies have shown that C1 esterase inhibitor has some protective benefit in severe acute pancreatitis[143]. Currently pharmacologic targets of the complement system are used in a variety of other diseases such as hereditary angioedema, paroxysmal nocturnal hemoglobinuria, and hemolytic uremic syndrome[144] that may permit more rapid translation.

CONCLUSION

Both pre-clinical and clinical studies (Tables 1 and 2) have

Table 1 Summary of pharmacologic agents studied in experimental acute pancreatitis

Pharmacologic agent	Animal model		Outcome assessment	Citations
	Species name	Mechanism of pancreatitis induction		
Anti-secretory agents				
Glucagon	Dog	Duodenal obstruction, pancreatic duct infusion of lactated ringer solution or pancreatic duct infusion of bile-trypsin solution	Not beneficial when compared to simple volume resuscitation	[15]
	Pig	Hemorrhagic pancreatitis induced by bile injection into pancreatic duct	Reduced mortality	[16]
Somatostatin	Rat	Taurocholate	No decrease in mortality	[21, 22]
Protease inhibitors				
Aprotinin	Dog	Hemorrhagic pancreatitis surgically induced	Prophylactic and therapeutic potential	[17-19]
Chlorophyll-a	Guinea pig	Taurocholate-induced necrotizing pancreatitis	Benefit in survival	[25-27]
Anti-inflammatory/ immunomodulators				
PGE therapy	Rat, mice	Taurocholate, CDE diet, or caerulein	Protective effect	[29-31]
Indomethacin	Rat	Olive oil or taurocholate	Beneficial particularly early in induction	[32-34]
Lipoxygenase inhibitor	Rat	Taurocholic acid	Protective effect	[35]
Steroid	Rat	Caerulein and taurocholate	Decreased inflammation and protective	[37-43]
IL-10	Rat, mice	Caerulein	Reduction in severity of disease	[44, 48-50]
Lexipafant	Rat, mice	Intraductal administration of 5% sodium taurodeoxychlolate or caerulein	Reduction in severity, SIRS, and bacterial translocation	[46, 47]
Hemin/panhematin/ biliverdin/CO/IL-22	Rat, mice	caerulein, taurocholate, or CDE diet	Protective and therapeutic effects	[61-67]
Anti-TNF alpha	Mice	caerulein, taurocholate, or CDE diet	Decreased inflammatory response and cell death	[72-75]
Anti-oxidants				
Tempol	Mice	carrageenan injected into pleural cavity	Decrease in inflammation and shock	[54]
Selenium	Rat	L-arginine hydrochloride	Reduction in pancreatic injury	[53, 145]

CO: Carbon monoxide; PGE: Prostaglandin E1; SIRS: Systemic inflammatory response syndrome; TNFα: Tumor necrosis factor α; CDE: Choline deficient ethionine-supplemented.

shown promising opportunities for novel pharmacologic therapy for acute pancreatitis that can supplement the traditional treatment involving supportive measures such as fluid resuscitation, nutritional support, pain control, and antibiotics as needed. Pre-clinical and clinical studies have shown promise in a variety of classes of therapies that include anti-secretory agents, protease inhibitors, immunomodulators and anti-inflammatory agents, and anti-oxidants. While some of the evidence for these therapies still remains inconclusive and hasn't been translated into current standard treatment care, there exists a tremendous potential therapeutic benefit as demonstrated in these studies. The immunomodulating pharmacologic therapies also have yet to be translated into standard clinical care for acute pancreatitis[67].

There are also new targets for pharmacologic therapy that can expand the potential therapies for acute pancreatitis. Strategies that alter the activity of key immune cells in the inflammatory cascade triggered by acute pancreatitis offer great potential[67]. Other molecular targets such as those that interfere with the kallikrein-kinin, proteolytic, and complement system as discussed with further development have the potential of being applied to acute pancreatitis as well in the future. In addition to expanding targets for pharmacologic therapy, existing therapies need to be better studied in clinical trials in the future.

Experimental pre-clinical studies have identified several therapies that have not proven to be effective in clinical trials and thus have not been translated to the clinical arena. One of the reasons for this discrepancy may be that in the animal models, the pharmacologic therapy is often administered prior to when pancreatic injury ensues thus providing evidence that the therapy can provide a protective but not necessarily therapeutic effect.

In the clinical studies, however, the medication of interest is often tested once the pancreatic injury has already occurred and the inflammatory cascade induced by acute pancreatitis has already initiated. In the future, better design of clinical trials that deliver the treatment earlier from symptom onset can maximize the drug's ability to interrupt the inflammatory cascade and yield better results. Clinical trials need to also be standardized with respect to eligibility criteria, supportive treatment approaches, and outcomes measured. Clinically meaningful primary and secondary outcomes such as mortality, organ failure, SIRS, pancreatic necrosis, and local complications, length of hospital stay, requirement for pain medications, quality of life, and cost of care should be clearly outlined.

Despite the inconclusive evidence in therapeutic benefit seen with many of the pharmacologic therapies for acute pancreatitis studied thus far, there exists great need and promise in the development of effective pharmaco-

Table 2 Summary of pharmacologic agents studied in clinical acute pancreatitis

Pharmacologic agent	Study design	Sample size	Outcome assessment					Citations
			Decreased SIRS	Decreased organ failure	Decreased length of stay	Decreased mortality	Other	
Anti-secretory agents								
Glucagon	RCT	22-69	Not reported	Not reported	No	No		[76-82]
Atropine	RCT	51	Not reported	Not reported	No	No		[83]
Calcitonin	RCT	94	Not reported	Not reported	Not reported	No	↓ pain, earlier normalization of labs	[84-86]
Somatostatin	RCT/meta-analysis	50-703	Not reported	Indeterminate (no effect on multi-organ failure but ↓ local complications)	Indeterminate	Indeterminate	↓ pancreatic abscess and necrosis, ↓ local inflammation	[90-95]
Octreotide	RCT/meta-analysis	19-948	Yes	Yes	Indeterminate	Indeterminate		[97-99]
Protease inhibitors								
Aprotinin	RCT	48-105	Not reported	No	Yes	No	↓ pancreatic necrosis, ↓ complement activation	[101-105]
Gabexate mesilate	RCT/meta-analysis	42-898	Not reported	No	No	No	CRAI ↓ hospitalization stay and SIRS	[108-113]
Nafomostat	RCT	51-78	Not reported (↓ pancreatic necrotic tissue infection)	Not reported	Not reported	Yes	Only CRAI + abx has benefit	[114-116]
Immunomodulators								
Lexipafant	RCT	50-290	Yes	Yes	Not reported	Yes	↓ local complications (pancreatic abscess, pseudocyst)	[117-119]
Dotrecogin alfa	RCT	32	Yes	No	Not reported	No		[122, 123]
Acetylcysteine, selenium, vitamin C combinations	RCT	39-53	Indeterminate (↓ CRP but not sig)	No (trend toward ↑ MOF)	No	No		[132-134]
Glutamine	RCT/meta-analysis	505	Yes	Yes	No	Yes		[135, 136]

RCT: Randomised controlled trials; SIRS: Systemic inflammatory response syndrome; CRAI: Continuous regional arterial infusion; ERCP: Endoscopic retrograde cholangiopancreatography; MOF: Multiple organ failure; RCT: Randomized controlled trial.

logic therapy for acute pancreatitis. Better understanding of the pathophysiology of the disease and lessons learned from past clinical studies offer a great foundation upon which to expand such that the current management of pancreatitis largely characterized by supportive therapy can eventually be transitioned to not only preventive but also to reparative and effective therapy. Better characterization and standardization of the patient population, along with well controlled and adequately powered clinical studies tied to standardized outcomes, will ensure a reliable and valid assessment of the therapeutic role of preclinical tested agents.

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2014 ADVANCES IN PANCREATITIS

Autoimmune pancreatitis: Multimodality non-invasive imaging diagnosis

Stefano Crosara, Mirko D'Onofrio, Riccardo De Robertis, Emanuele Demozzi, Stefano Canestrini, Giulia Zamboni, Roberto Pozzi Mucelli

Stefano Crosara, Mirko D'Onofrio, Riccardo De Robertis, Emanuele Demozzi, Stefano Canestrini, Giulia Zamboni, Roberto Pozzi Mucelli, Department of Radiology, University Hospital GB Rossi, University of Verona, 37134 Verona, Italy
Author contributions: All the authors equally contributed to this paper.
Correspondence to: Mirko D'Onofrio, MD, Assistant Professor, Department of Radiology, University Hospital GB Rossi, University of Verona, Piazzale LA Scuro 10, 37134 Verona, Italy. mirko.donofrio@univr.it
Telephone: +39-45-8124140 Fax: +39-45-8277808

Abstract

Autoimmune pancreatitis (AIP) is characterized by obstructive jaundice, a dramatic clinical response to steroids and pathologically by a lymphoplasmacytic infiltrate, with or without a pancreatic mass. Type 1 AIP is the pancreatic manifestation of an IgG4-related systemic disease and is characterized by elevated IgG4 serum levels, infiltration of IgG4-positive plasma cells and extrapancreatic lesions. Type 2 AIP usually has none or very few IgG4-positive plasma cells, no serum IgG4 elevation and appears to be a pancreas-specific disorder without extrapancreatic involvement. AIP is diagnosed in approximately 2%-6% of patients that undergo pancreatic resection for suspected pancreatic cancer. There are three patterns of autoimmune pancreatitis: diffuse disease is the most common type, with a diffuse, "sausage-like" pancreatic enlargement with sharp margins and loss of the lobular contours; focal disease is less common and manifests as a focal mass, often within the pancreatic head, mimicking a pancreatic malignancy. Multifocal involvement can also occur. In this paper we describe the features of AIP at ultrasonography, computed tomography, magnetic resonance and positron emission tomography/computed tomography imaging, focusing on diagnosis and differential diagnosis with pancreatic ductal adenocarcinoma. It is of utmost importance to make an early correct differential diagnosis between these two diseases in order to identify the optimal therapeutic strategy and to avoid unnecessary laparotomy or pancreatic resection in AIP patients. Non-invasive imaging plays also an important role in therapy monitoring, in follow-up and in early identification of disease recurrence.

Core tip: In this paper we describe the features of autoimmune pancreatitis (AIP) at ultrasonography, computed tomography, magnetic resonance and positron emission tomography/computed tomography imaging, focusing on diagnosis and differential diagnosis with pancreatic ductal adenocarcinoma, which has a similar imaging appearance but a completely different therapeutic management. It is of utmost importance to make an early correct differential diagnosis between these two diseases in order to identify the optimal therapeutic strategy and to avoid unnecessary laparotomy or pancreatic resection in AIP patients. Non-invasive imaging plays also an important role in therapy monitoring, in follow-up and in early identification of disease recurrence.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a distinct form of pancreatitis frequently characterized by obstructive jaundice and by a dramatic clinical response to steroids; pathologically, it is characterized by a lymphoplasmacytic infiltrate, with or without a pancreatic mass. The term AIP was first used in 1995 by Yoshida *et al*[1] to describe a type of chronic pancreatitis associated with a Sjogren-like syndrome. Recently AIP was divided into type 1 and type 2 which have distinct histopathology, clinical features and different diagnostic criteria[2-4].

Type 1 AIP is also called lymphoplasmacytic sclerosing pancreatitis (LPSP) or AIP without granulocyte epithelial lesions (GEL) and pathology of the pancreas shows four characteristic features[3-7]: (1) Dense periductal infiltration of plasma cells and lymphocytes; (2) Peculiar storiform fibrosis; (3) Venulitis with lymphocytes and plasma cells often leading to obliteration of the affected veins; and (4) Abundant IgG4-positive plasma cells.

Type 1 AIP seems to be the pancreatic manifestation of an IgG4-related systemic disease, characterized by elevated IgG4 serum levels, infiltration of IgG4-positive plasma cells and extrapancreatic lesions (*e.g.,* sclerosing cholangitis, sclerosing sialoadenitis and retroperitoneal fibrosis). This form of AIP presents predominantly with obstructive jaundice in elderly male subjects; both pancreatic and extrapancreatic manifestations respond to steroid therapy. The clinical diagnosis of LPSP can be made without need for a histology sample[3-7].

Type 2 AIP is also defined idiopathic duct-centric pancreatitis (IDCP) or AIP with GEL[3-10]. It shares with LPSP some histopathological features, such as periductal lymphoplasmocytic infiltrates and storiform fibrosis. A characteristic feature of IDCP are GELs: intraluminal and intraepithelial neutrophils, leading to destruction and obliteration of pancreatic duct lumen. IDCP usually has none or very few IgG4-positive plasma cells, no serum IgG4 elevation and appears to be a pancreas-specific disorder without extrapancreatic involvement. Approximately 30% of reported cases of IDCP are associated with inflammatory bowel disease, frequently ulcerative colitis. Patients with IDCP are, on average, a decade younger than LPSP patients and the disease does not show a sex preference. Because IDCP patients are seronegative and lack other organ involvement, definitive diagnosis requires pancreatic histology[3-7,11].

DIAGNOSTIC CRITERIA

In 2011, the International Consensus Diagnostic Criteria (ICDC)[3] were developed by the International Association of Pancreatology after a review of existing criteria, including Japanese Pancreas Society criteria (JPS 2002, 2006)[12], HISORt criteria of the Mayo Clinic (2006, 2009)[13,14], Korean criteria (2007)[15], Asian criteria (2008)[16] and Mannheim criteria (2009)[17]. ICDC are composed of five cardinal features such as imaging of the pancreatic parenchyma on computed tomography

(CT) and magnetic resonance (MR) and duct on endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance cholangiopancreatography (MRCP), serology, other organ involvement, histology and response to steroid therapy[3]. ICDC can be used to diagnose type 1 and type 2 AIP independently[3].

EPIDEMIOLOGY

The true incidence of AIP is unknown. AIP was diagnosed in approximately 2%-6% of patients that underwent pancreatic resection for suspected pancreatic cancer[18,19]. In Japan the incidence of AIP was reported to be 0.82 per 100000 population[20].

PATHOPHISIOLOGY

The precise pathogenesis of AIP has not been elucidated. It is still unclear if IgG4 plays a direct pathogenic role in developing AIP or if their presence is an epiphenomenon[21,22]. Molecular mimicry by a microbial pathogen, which leads to a cross reaction with endogenous antigens, has been postulated as a cause of many autoimmune conditions including AIP[23,24].

CLINICAL ISSUES

The clinical presentation of AIP can be divided into acute and subacute phase. In the acute phase, the classic presentation of AIP is that of obstructive jaundice with abdominal imaging showing pancreatic enlargement[2-5,13]. Thus it is imperative to differentiate AIP from pancreatic cancer, especially in localized forms. Less commonly AIP presents with mild abdominal pain and elevated pancreatic enzymes, which may also be consistent with acute pancreatitis. In the subacute phase, after initial treatment, AIP can present with pancreatic atrophy and steatorrhea resembling chronic pancreatitis. Severe unremitting abdominal pain requiring narcotic pain medication is hardly ever present[3]. The presence of such severe pain should prompt a re-evaluation of the diagnosis. Diabetes mellitus (DM) is seen in up to 50% of patients with AIP and resolves in a proportion of patients with corticosteroid therapy[20,25].

OTHER ORGAN INVOLVEMENT

As previously stated, type 1 AIP is the pancreatic manifestation of a systemic disease. The involvment of other organs can lead to characteristic symptoms, such as xeroftalmia and xerostomia (Sjogren-like syndrome), jaundice (bile ducts involvement), and swelling in the groin (regional lymphoadenopathy). Other organ involvement that can be seen on abdominal imaging includes retroperitoneal fibrosis and renal involvement (interstitial nephritis). When present, these signs strengthen the diagnosis of AIP, and also prompt the histologic confirmation of AIP itself[5,26-28]. Less commonly, gallbladder and gastric involvement have also been described[29]. Symptoms
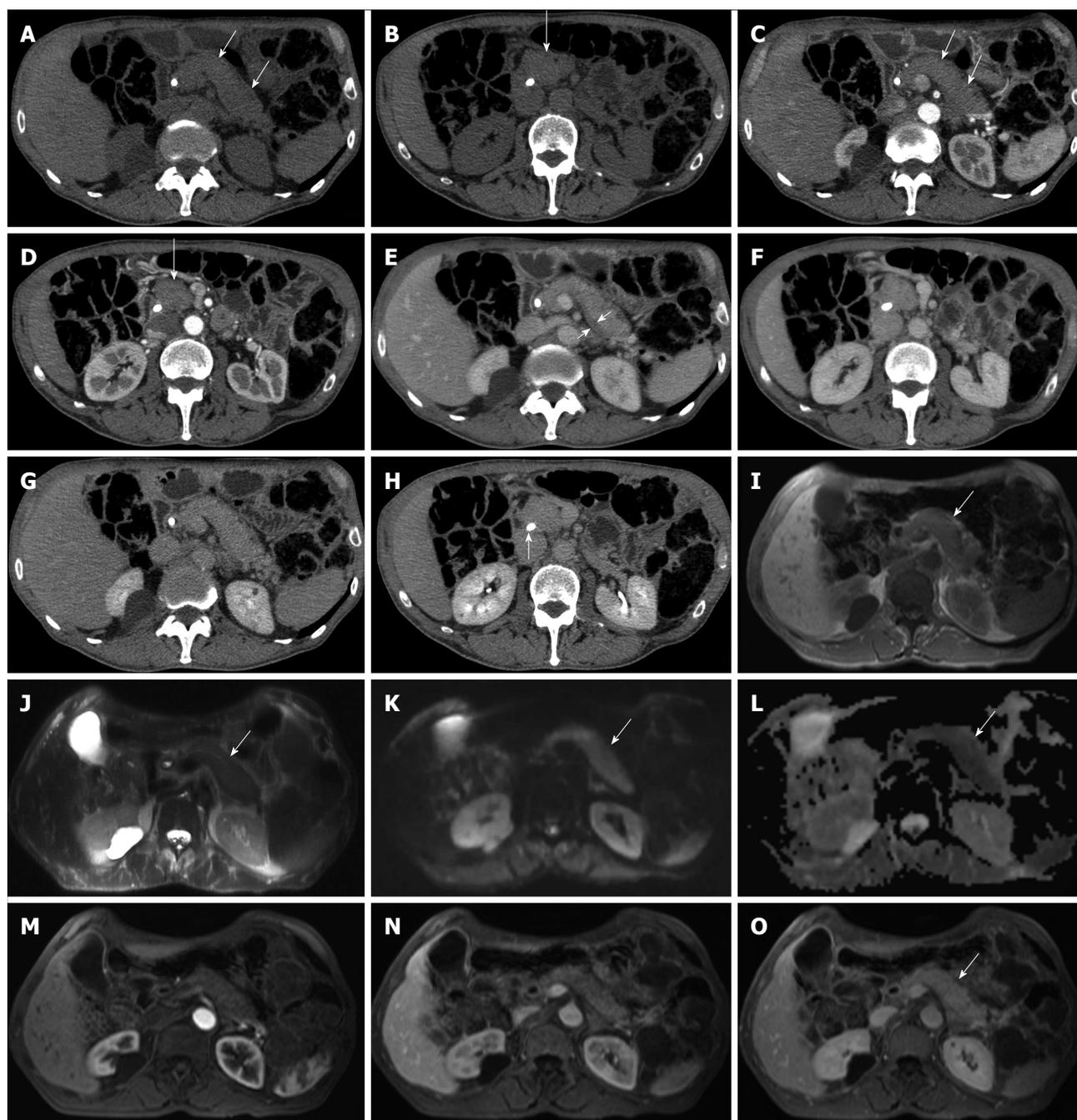
Figure 1 Diffuse-type autoimmune pancreatitis. A-H: Computed tomography: the pancreas appears diffusely enlarged (arrows in A-D) with a hypodense peripancreatic rim, better visible in the venous phase (arrow in E). The lesion shows fair enhancement resulting almost isodense in the delayed phase (G-H). A plastic biliary endoprothesis is visible in the common bile duct (arrow in H); I-O: Magnetic resonance: the entire organ is slightly hypointense on T1-weighted images (arrow in I) and slightly hyperintense on T2-weighted images (arrow in J), with diffusion coefficient restriction (arrows in K and L) with intermediate-high b values. At dynamic examination the pancreatic lesion presents fair enhancement resulting almost isodense in the delayed phase (arrow in O).

related to other organ involvement often improve with treatment and can be useful for the assessment of treatment response[4].

IMAGING

There are three recognized patterns of AIP: diffuse, focal and multifocal. Diffuse disease is the most common type, with a diffuse, "sausage-like" pancreatic enlargement with sharp margins, loss of the lobular contours, and absence of pancreatic clefts (Figure 1)[30,31]. Focal disease is less common than diffuse disease and manifests as a focal mass, often within the pancreatic head, an appearance that may mimic that of a pancreatic malignancy (Figure 2). Focal disease tends to be relatively well demarcated and, when present, upstream dilation of the main pancreatic duct is typically milder than what is observed in patients with pancreatic carcinoma. In some patients with focal AIP, only the dorsal pancreas or the pancreatic tail is involved[32]. Multifocal involvement can also be evident.
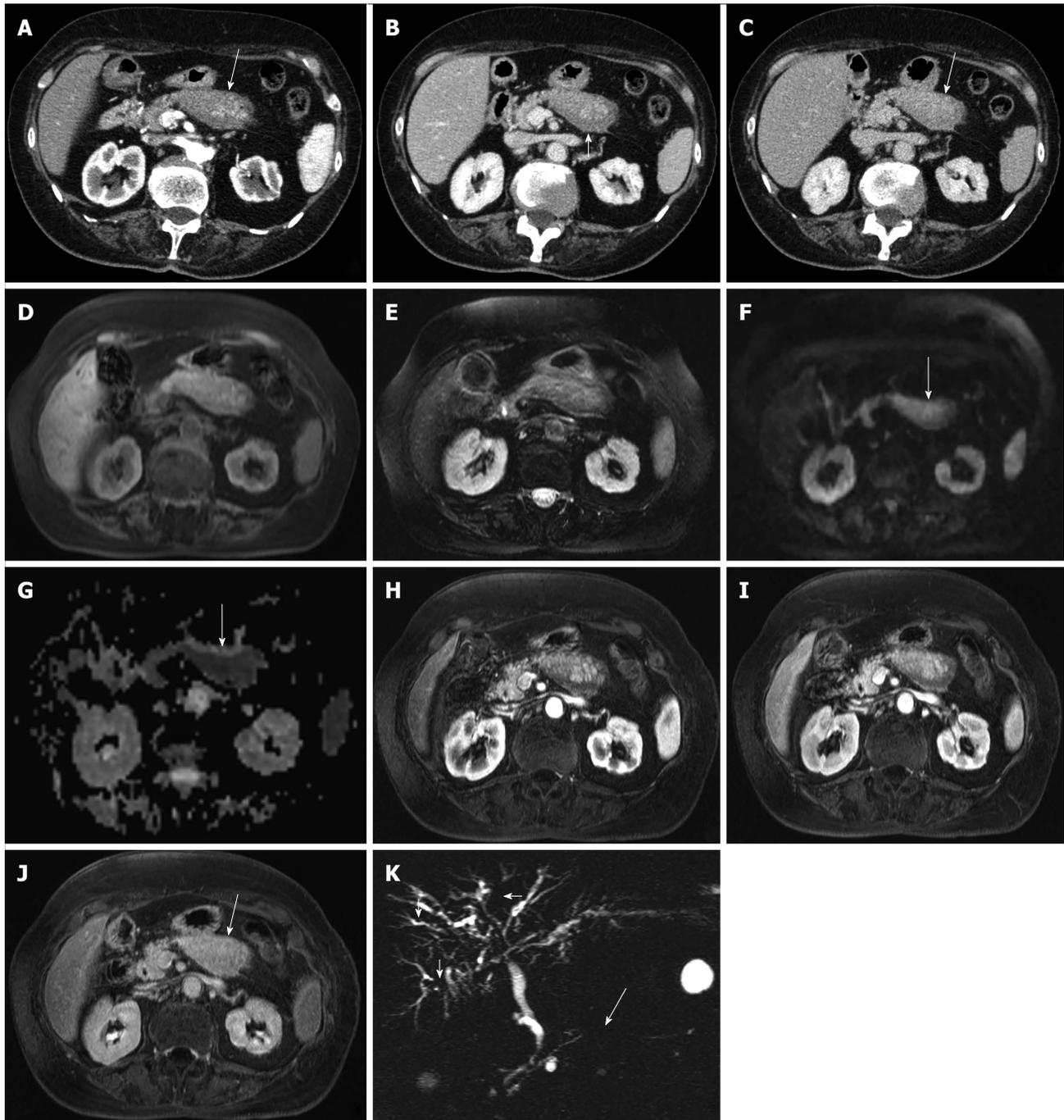
Figure 2 Focal-type autoimmune pancreatitis. A-C: Computed tomography: the body of the pancreas appears focally enlarged (arrow in A) with a hypodense peripancreatic rim, better visible in the venous phase (arrow in B). The lesion shows fair enhancement resulting almost isodense in the delayed phase (arrow in C); D-K: Magnetic resonance: the affected portion of the pancreas is slightly hypointense on T1-weighted fat-saturated (arrow in D) images and slightly hyperintense on T2-weighted fat-saturated images (E), with diffusion coefficient restriction (arrows in F-G) with intermediate-high *b* values. At dynamic examination the pancreatic lesion shows fair enhancement resulting almost isodense in the delayed phase (arrow in J). At magnetic resonance cholangiopancr-eatography the main pancreatic duct shows a focal stenosis (long arrow in K) without upstream dilation. The intrahepatic bile ducts present irregular slightly stenotic portions (short arrows in K), due to involvement in the autoimmune process.

Transabdominal ultrasonography

Conventional ultrasonography (US) is often the first imaging exam performed in presence of any abdominal symptom since it is noninvasive, inexpensive, easy to perform and widely available. US of diffuse form of AIP shows a diffusely enlarged and hypoechoic pancreatic parenchyma. In the focal and multifocal forms of AIP only the affected regions of the pancreas appear hypoechoic. This appearance, however, is not specific and includes many features commonly seen in other types of acute and chronic pancreatitis.

At color-Doppler, the enlarged pancreas can show hypervascularity[33]. Conventional US is often not able to show the irregular focal or diffuse narrowing of the main

pancreatic duct or of the intrahepatic bile duct, which represents one of the main diagnostic criteria[3]. Contrast-enhanced US can successfully visualize fine vessels in pancreatic lesions and may play a pivotal role in the depiction and differential diagnosis of pancreatic tumors[34].

Computed tomography

Cross sectional pancreatic imaging is the cornerstone to the diagnosis of AIP. Quadriphasic abdominal CT and MR examinations are the imaging modalities of choice to diagnose AIP. CT scan is of utmost importance in diagnosing AIP and in confirming or ruling out pancreatic cancer. Classic features of diffuse AIP at CT are a diffusely enlarged hypodense sausage-shaped pancreas with sharp and smooth borders; decreased enhancement of the pancreatic gland in the early phase and moderate and persisting delayed enhancement in the late phase are found in 90% of the cases, a finding due to fibrosis[3,14,35,36]. Supplementary findings include a hypodense capsule-like peripheral rim with subtle delayed enhancement[35] sorrounding the pancreas (12%-40% of cases), which is believed to represent fluid, flegmon or fibrous tissue due to inflammatory changes of the peripancreatic tissues[30,31,35,36].

When AIP presents as a focal enlargement of the pancreas, it is more often located in the pancreatic head[37]. A segmental enlargement of the pancreas is seen in 30%-40% of the patients with AIP. The enlarged segment of the pancreas is typically isoattenuating or hypoattenuating to the spared, non-enlarged portion of parenchyma and may be indistinguishable from pancreatic cancer[30,36,38,39].

Unlike from many other causes of pancreatitis, peripancreatic stranding is usually minimal in AIP but can occur[40]. Involution of the pancreatic tail and regional lymphoadenopathy may also be seen[37]. Segmental or diffuse narrowing of the main pancreatic duct, involvement of the distal common bile duct, and multiple cholangitis-like bile duct strictures have been described but are better depicted on MR or MRCP or by means of ERCP than at CT[41,42].

Atrophic pancreatic parenchyma represents a late burnt-out phase of the disease[30,36]. This appearance may also persist after steroid therapy.

Magnetic resonance

At MR, AIP shows a similar appearance to CT: the pancreas is diffusely, focally or multifocally enlarged, and the involved portion is hypointense on T1-weighted images, slightly hyperintense on T2-weighted images, and has heterogeneously diminished enhancement in the early phase and delayed enhancement in the late phase of contrast enhancement[30,35,43,44]. The capsule-like rim described at CT is usually hypointense on both T1 and T2-weighted images, and has delayed moderate enhancement on contrast-enhanced MR[35,44].

Other imaging hallmarks of AIP include multiple narrowings of the main pancreatic duct or an irregularly nar-

rowed main pancreatic duct in the affected segment[12,30]. Narrowing of the main pancreatic duct in AIP is usually longer than 3 cm in the diffuse form of AIP[45]. MRCP is a less invasive and more easily performed technique than ERCP but Kamisawa *et al*[45] stated that it cannot completely replace ERCP for diagnosing AIP, since narrowing of the main pancreatic duct in AIP cannot be always visualized on MRCP as clearly as on ERCP and in some studies[46] the narrowed main pancreatic duct could not be seen at MRCP at all. However, MRCP findings of a segmental or skipped non-visualized main pancreatic duct accompanied by less upstream main pancreatic duct dilatation than what is usually seen with adenocarcinoma may suggest the presence of focal AIP[45,47,48]. The irregular narrowing of the main pancreatic duct, which is usually longer than the stenosis caused by pancreatic adenocarcinoma, is one of the useful findings to differentiate focal AIP from pancreatic adenocarcinoma[49,50] together with the absence of upstream duct dilation, since ductal stenosis is not as strict as the one of adenocarcinoma[43,51]. A study by Muhi *et al*[39] revealed that 4 mm is the optimal cutoff value of ductal dilation to differentiate between focal AIP and pancreatic cancer[39]. Moreover, according to some studies, secretin stimulation during MRCP is of key importance to differentiate focal AIP and pancreatic adenocarcinoma, since the main pancreatic duct in focal AIP is not completely obstructed and tends to penetrate the mass after secretin administration, with the so-called "penetrating duct sign", which has been described to be highly specific for benign strictures[52,53]. Another useful finding among AIP ductal abnormalities, not frequently seen in pancreatic cancer, is the presence of secondary pancreatic ducts deriving from the narrowed portion of the main pancreatic duct in AIP patients.

Bile duct abnormalities can be also recognized. These include smooth narrowing of the intrapancreatic portion of the common bile duct[40,43], or irregularity and stricturing of the intra- and extra-hepatic bile ducts with features similar to those seen in primary sclerosing cholangitis. Enhancing duct wall thickening is also a recognized feature and, less commonly, intra-hepatic bile duct dilation may also be observed[40,43].

Diffusion-weighted magnetic resonance imaging (DWI) has been increasingly used to evaluate diseases involving abdominal organs. Quantitative measurement of the diffusivity of water molecules in various tissues are described by the apparent diffusion coefficient (ADC) value. ADC is correlated to blood microcirculation, as well as molecular diffusion of water, frequently altered in various disease processes due to changes in physiological and morphological characteristics, such as cell density and tissue viability. Decreased ADC values correlate with increased lesion cellularity and total nuclear area, both restricting water diffusion. In general, malignant tumors have higher cellularity than benign lesions[54]. At DWI, AIP and pancreatic cancer are both detected as high signal intensity areas at high *b*-values images; however, pancreatic cancer usually present as a solitary area, while

diffuse or multiple high-intensity areas are suggestive for AIP[55,56]. A longitudinal high intensity area also suggests AIP more than pancreatic cancer[55]. It has been found that mean ADC values are significantly lower in AIP than in pancreatic cancer, which has ADC values lower than normal pancreatic parenchyma[57,58]. Muhi *et al*[39] found that the optimal ADC cutoff value (100% sensitivity and 89% specificity) for differentiating mass-forming AIP from pancreatic carcinoma would be 0.88×10^{-3} mm^2/s. Similarly Kamisawa *et al*[55] found ADC values to be significantly lower in AIP patients ($1.012 \times 10^{-3} \pm 0.112 \times 10^{-3}$ mm^2/s) than in pancreatic cancer patients ($1.249 \times 10^{-3} \pm 0.113 \times 10^{-3}$ mm^2/s). The reason of these findings resides in the anatomo-pathological features of these lesions: although cancer cell infiltration with desmoplastic stroma is the typical histopathological feature of pancreatic cancer, the cellularity of the dense lymphoplasmocytic infiltrate in AIP is greater than that of pancreatic cancer, therefore increased cellularity in AIP induce lower ADC values in AIP than in pancreatic cancer[12,22,28].

[18]F-fluorodeoxyglucose positron emission tomography/CT

Many patients with AIP are likely to be among those who receive fluorodeoxyglucose positron emission tomography (FDG-PET) because of suspected pancreatic cancer. However, even FDG-PET cannot always differentiate between these two lesions because inflammatory foci in the pancreas also accumulate FDG with the same avidity as a pancreatic neoplasm[59,60]. AIP causes intense FDG uptake by the pancreas[61,62]. Ozaki *et al*[63] showed FDG uptake in all AIP patients of their series and in 73.1% of pancreatic cancer patients. In contrast, previous studies had found that the sensitivity of FDG uptake to be higher (96%) in patients with pancreatic cancer[60], and lower (83%) in those with AIP[62]. Typical FDG-PET findings for AIP[63,64] are heterogeneous longitudinal accumulation and multiple localizations, whereas those for pancreatic cancer are nodular homogeneous accumulation, and solitary localization. When FDG accumulation in AIP is focal, differentiation from pancreatic cancer can be difficult. The longitudinal FDG uptake found in AIP is due to diffuse distribution of the inflammatory process, and FDG uptake by inflammatory cells possibly results in heterogeneous accumulation because of the scattered distribution of inflammatory cells. However, diffuse-type pancreatic cancer may also show a similar longitudinal shape, although such cases are rare. FDG uptake by extrapancreatic organs may assist in differentiating the two conditions.

DIFFERENTIAL DIAGNOSIS

The most common presentation of AIP is with obstructive jaundice and pancreatic enlargement that mimics the presentation of pancreatic cancer[14], and 5%-21% of patients undergoing resection for suspected pancreatic cancer have a final diagnosis of benign disease, including AIP[65,66]. As mentioned above, pancreatic enlargement can be focal or diffuse: when AIP presents as focal pancreatic enlargement with mass effect differentiating AIP from pancreatic cancer at imaging can be challenging. Since AIP responds extremely well to steroid therapy, it is of utmost importance to differentiate it from pancreatic cancer to avoid unnecessary laparotomy or pancreatic resection.

Obstructive jaundice caused by pancreatic cancer typically progresses steadily, whereas AIP jaundice sometimes fluctuates or, in rare cases, improves spontaneously[4,55,67].

Although false positive elevation of IgG, IgG4 and other antinuclear antibodies can be seen in pancreatic cancer[3], a marked elevation of serum IgG4 (> 2 times the upper limit of normal) is strongly suggestive of AIP in the setting of obstructive jaundice/pancreatic mass[3].

At CT the "sausage-like" appearance of the pancreas is the typical finding in AIP and is rarely seen in pancreatic cancer[56]. Enhancement of an enlarged pancreas on the delayed phase of CT and MR is characteristic of AIP[56]. As fibroinflammatory changes involve the peripancreatic adipose tissue, a capsule-like rim surrounding the pancreas is specifically detected in some AIP patients[30,32,44].

Some studies[52,68] state that MRCP findings such as skipped strictures of the main pancreatic duct without significant upstream dilation and the "penetrating duct sign" are most frequently seen in AIP patients.

As mentioned above, both AIP and pancreatic cancer are detected as high signal intensity areas on DWI images[55,56]. However, these areas are differently shaped, being diffuse, solitary or multiple in AIP, whereas all patients with pancreatic cancer have solitary areas[55,56]. In addition ADC values have been demostrated to be significantly lower in AIP than in pancreatic cancer[55,56].

Morover, while clarifying the differential diagnosis between AIP and pancreatic cancer, it has to be clear that the presence of other organ involvement and responsiveness to steroids are both highly suggestive of AIP.

The differential diagnosis between diffuse AIP and lymphoma may be difficult, since both entities determine enlargement of the pancreatic parenchyma and appear hypoattenuating in the pancreatic phase. Therefore, the differential diagnosis is based on ancillary findings, such as retroperitoneal and pelvic enlarged lymphnodes, splenic lesions, or both; when necessary fine needle aspiration or core biopsy are performed[69].

TREATMENT

Both subtypes of AIP are exquisitely sensitive to steroid therapy. The response to corticosteroid therapy can be both diagnostic and therapeutic. When typical imaging features and collateral evidence for AIP are absent and pancreatic cancer has been reliably ruled out, a steroid trial of oral prednisone for 2 wk can be started. Response to steroids is based on objective data such as radiologic evidence a dramatic decrease in the pancreatic mass or other organ involvement, resolution of the obstructive jaundice without biliary stenting, and normalization of

liver function tests. If there is no such improvement or if the cancer antigen 19.9 level is rising, then the diagnosis of AIP should be reconsidered.

Once the diagnosis of AIP has been established, the best initial treatment is oral prednisone for 4 wk. Beginning at week 4, with continued objective response to therapy, the dose should be tapered.

Up to 40% of patients (mostly with type 1 AIP) will have disease relapse after the first course of corticosteroid therapy[70,71]. Proximal bile duct involvement can be a predictor of disease relapse.

The most severe cases of AIP are not responsive to pharmacologic treatment and requires surgical intervention. In cases with focal involvement of the pancreatic head region, pancreatico-duodenectomy is most frequently performed. Focal forms of AIP with body-tail involvement are treated with distal spleno-pancreatectomy. Diffuse forms of AIP, not responsive to corticosteroid therapy can require total pancreatectomy[72].

FOLLOW-UP

Laboratory findings and clinical evaluation are of great importance in the follow-up of patients with AIP, but imaging, mainly performed with CT and MR, plays a pivotal role.

Corticosteroid therapy induces the resolution of pancreatic changes. The gland swelling decreases, the physiological lobularity of the pancreatic contour is again visible and the other pancreatic (parenchymal eterogeneity and tail retraction) and peripancreatic (peripancreatic fat stranding and hypodense halo) changes improve. This improvement can be partial or complete and sometimes the pancreas can become slightly atrophic[51,73]. In patients with partial response retraction of the pancreatic tail can persist or a focal mass-like swelling can still be visible after therapy.

Manfredi *et al*[69] reported that the enhancement pattern returned to its normal appearance in the majority of patients, with the previously affected parenchyma resulting isoattenuating to the spleen or the unaffected adjacent parenchyma in the pancreatic phase.

At MR, steroid treatment resulted in significant changes in signal intensity on both T1- and T2-weighted images as compared to the pre-treatment images: the previously affected pancreatic parenchyma regains its physiological signal intensity in the majority of treated patients[46]. In more than 65% of the cases the affected parenchyma presents a post-therapy physiological contrastographic behaviour, resulting isointense to the non-affected parenchyma in every dynamic phase[46]. After steroid therapy, the main pancreatic duct has normal caliber, persisting narrowed only in a small percentage of patients, infrequently with a slight upstream dilation[46,69]. Therapy induces also the regularization of the common bile duct[46,69].

MR is also useful in the post-therapy follow up with DWI sequences: after steroid therapy, high intensity areas

on DWI disappear or are markedly decreased in the same way as the pancreatic enlargement. The reduced ADC values of the inflammatory lesions usually increase to nearly those of normal pancreas. Remaining or recurring areas of low ADC indicate disease recurrence[55,74].

Disease recurrence occurs more frequently in young patients with focal forms of AIP. It tends to be morphologically similar to the previous presentation of the disease and with the same imaging features. Rarely AIP recurrence presents as diffuse form of the disease[69,75].

CONCLUSION

In conclusion, in the light of the recent literature and the latest published guidelines, it is clear that noninvasive imaging modalities play a progressively more important role in the diagnosis of AIP. Imaging is also of utmost importance for differential diagnosis, therapy monitoring, follow-up and early identification of disease recurrence.

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2014 ADVANCES IN PANCREATITIS

Acute recurrent pancreatitis: Etiopathogenesis, diagnosis and treatment

Pier Alberto Testoni

Pier Alberto Testoni, Division of Gastroenterology and Gastrointestinal Endoscopy, Vita-Salute San Raffaele University, Scientific Institute San Raffaele, 20132 Milan, Italy
Author contributions: Testoni PA contributed to the manuscript.
Correspondence to: Pier Alberto Testoni, MD, Division of Gastroenterology and Gastrointestinal Endoscopy, Vita-Salute San Raffaele University, Scientific Institute San Raffaele, via Olgettina 60, 20132 Milan, Italy. testoni.pieralberto@hsr.it
Telephone: +39-2-26432756 Fax: +39-2-26433491
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Abstract

Acute recurrent pancreatitis (ARP) refers to a clinical entity characterized by episodes of acute pancreatitis which occurs on more than one occasion. Recurrence of pancreatitis generally occurs in a setting of normal morpho-functional gland, however, an established chronic disease may be found either on the occasion of the first episode of pancreatitis or during the follow-up. The aetiology of ARP can be identified in the majority of patients. Most common causes include common bile duct stones or sludge and bile crystals; sphincter of oddi dysfunction; anatomical ductal variants interfering with pancreatic juice outflow; obstruction of the main pancreatic duct or pancreatico-biliary junction; genetic mutations; alcohol consumption. However, despite diagnostic technologies, the aetiology of ARP still remains unknown in up to 30% of cases: in these cases the term "idiopathic" is used. Because occult bile stone disease and sphincter of oddi dysfunction account for the majority of cases, cholecystectomy, and eventually the endoscopic biliary and/or pancreatic sphincterotomy are curative in most of cases. Endoscopic biliary sphincterotomy appeared to be a curative procedure *per se* in about 80% of patients. Ursodeoxycholic acid oral treatment alone has also been reported effective for treatment of biliary sludge. In uncertain cases toxin botulin injection may help in identifying some sphincter of oddi dysfunction, but this treatment is not widely used. In the last twenty years, pancreatic endotherapy has been proven effective in cases of recurrent pancreatitis depending on pancreatic ductal obstruction, independently from the cause of obstruction, and has been widely used instead of more aggressive approaches.

Core tip: Acute recurrent pancreatitis still represents a challenging disease. In the recent years a significant improvement has been achieved in the knowledge of aetiopathogenesis and factors involved in the occurrence of disease because of advanced diagnostic tools as magnetic resonance cholangiopancreatography with secretin test, endoscopic ultrasonography and botulin toxin injection of sphinc ter of oddi. The review reports an updated diagnostic and therapeutic flow-chart flow-chart, and recent data on clinical outcomes.

INTRODUCTION

Acute recurrent pancreatitis (ARP) is a clinical condition characterized by repeated episodes of acute pancreatitis; ARP is therefore diagnosed retrospectively by clinical definition after at least the second episode of acute pancreatitis. The term ARP was reported in the first Marseille classification of pancreatitis[1] which clearly distinguished

ARP from chronic pancreatitis, and then in the classification of TIGAR-O[2], so-called from the acronym of the major predisposing risk factors. However, the term was eliminated in the revised classifications of Marseille[3] and Marseille-Rome[4] because of the difficulties of distinguishing between episodes of acute pancreatitis occurring in a normal pancreas or in chronic pancreatitis.

Pancreatitis generally recurs in a normal morpho-functional gland and is characterized by self-limited edematous changes in the pancreas. Acute episodes are generally mild to moderate, requiring 3-10 d in hospital; in some cases, pancreatic-like pain, with serum amylase and/or lipase elevation, lasts only a few hours and the patient recovers without hospitalization. Minor pancreatic lesions suggesting a chronic disease may be found in some cases, either at the first episode of pancreatitis or during the follow-up. This suggests that recurrent episodes of acute pancreatitis may complicate the course of chronic subclinical pancreatitis, meaning they are the clinical expression of chronic pancreatitis diagnosed in an early phase, or otherwise they may themselves induce chronic lesions as a consequence of repeated damage. Whether or not recurring bouts of pancreatitis in a morphologically normal pancreas can lead to chronic pancreatitis is still an open question, because only few, empirical data indicate whether, how often, and in which patients recurrent pancreatitis progresses to the chronic disease.

Alcohol and, more recently, smoking have been reported as the most frequent factors associated with the progression toward chronic disease. Ammann *et al*[5,6] reported a rate of chronic pancreatitis of about 80% over a 15-year period in a series of patients with recurrent pancreatitis and alcohol consumption. Four recent studies examined the progression to chronic pancreatitis in patients with recurrent pancreatitis[7-10]; progression to chronic disease was reported in from 4.0% to 32.3% of cases. However, only the most recent study[10] simultaneously assessed alcohol consumption, smoking, bile stone disease, and unknown etiology. This study was a population-based study, carried out in a very large series of patients recruited over a ten-year period and followed up for a median of 40 mo; chronic pancreatitis was related to alcohol, other causes including smoking, unknown causes, and bile stone disease in respectively 28%, 1%, 10%, and 6% of cases. These figures confirm that, excluding cases with a history of significant alcohol consumption, there is underlying chronic pancreatic disease in about one fourth of cases of recurrent acute pancreatitis of either known or unknown etiology and it can render ineffective a therapy that removes the recognized possible causes of acute pancreatitis.

In our series of 33 patients with pancreas divisum and a history of ARP, either suffering or not from pancreatitis episodes in the year preceding enrolment in the study and followed for five years, endoscopic ultrasound findings consistent with chronic pancreatitis were seen in similar proportions of patients undergoing endoscopic therapy and in the observation group (63.2% and 57.1%, respec-

tively). However, among patients undergoing endoscopic therapy, chronic pancreatitis findings were significantly less frequent in those whose treatment was successful. Dorsal duct dilation did not significantly affect the factors suggesting chronic pancreatitis in either group, confirming that factors other than ductal dilation may be involved in chronic disease in this category of patients.

Several factors play an etiologic role in ARP; in fact, any cause of acute pancreatitis can lead to recurrent episodes if it is not corrected. The etiology of ARP can be identified in the majority of patients and causes can be mechanical, inherited, autoimmune, metabolic, and drug-induced; parasites, vascular disorders, and toxic substances may also induce episodes of acute pancreatitis. The most common causes include common bile duct stones or sludge and bile crystals; sphincter of oddi dysfunction; anatomical variants of the pancreatic ductal system, common bile duct or pancreatico-biliary junction interfering with pancreatic juice outflow; obstruction of the main pancreatic duct or pancreatico-biliary junction; genetic mutations; alcohol consumption.

However, despite today's diagnostic technology, the etiology of ARP remains unknown in up to 30% of cases: in these cases the term "idiopathic" is used. The number of these cases diagnosed as "idiopathic", however, is decreasing as our understanding and diagnostic accuracy improve.

ETIOLOGY

Mechanical factors may induce episodes of acute pancreatitis by obstructing pancreatic juice outflow into the duodenum, with consequent intraductal hypertension, or inducing bile reflux into the main pancreatic duct, with intrapancreatic activation of zymogens (a theory proposed by Opie since 1901 for gallstone pancreatitis)[11]. Conditions that induce mechanical obstruction are either acquired or congenital and may be located at the level of the bilio-pancreatic junction, or main pancreatic duct.

Acquired conditions

Gallstone disease represents the most common condition associated with ARP in western countries. In bile duct stone disease, gallstones or bile sludge may induce acute pancreatitis either as a consequence of an impacted stone that obstructs the main pancreatic duct at the level of Vaters' papilla (a rare event), or because of transient papillary edema or papillary orifice relaxation following the passage of stones, that can obstruct the pancreatic juice flow or favor duodenopancreatic reflux, respectively. Transient ampullary obstruction may allow bile to reflux into the pancreas, even if the pressure in the main pancreatic duct in normal conditions is generally higher than in the common bile duct. Bile reflux into the pancreatic ductal system is facilitated if there is a common channel at bilio-pancreatic junction. The common channel theory, although debated, has been confirmed in two studies carried out in patients with recent episodes of gallstone-

induced acute pancreatitis who had undergone surgery; these studies documented the presence of amylase in the bile collected by a T-tube inserted into the common bile duct, suggesting there might be a functional channel between the two ductal systems[12,13].

The gallstone disease may also be manifested only by: (1) microlithiasis (stones less that 2 mm in diameter), that can be seen or suspected mainly at endoscopic ultrasound (EUS) or *endoscopic* retrograde cholangio-pancreatography (ERCP); (2) gallbladder sludge, that generally in normal conditions can only be visualized by EUS[14]; and (3) calcium carbonate, cholesterol monohydrate and calcium bilirubinate crystals, that can be detected only on microscopic examination of centrifuged bile aspirated from the duodenum or common bile duct in 36%-67% of patients[15-22]. However, microscopic bile crystals could merely indicate the presence of undetected stones, rather than cause *per se* an acute pancreatitis. In fact, either long-term ursodeoxycholic acid (UDCA) therapy, or cholecystectomy, or endoscopic biliary sphincterotomy have been found to prevent further episodes of 'idiopathic' pancreatitis in several series of patients without evidence of bile duct stones, confirming the role of occult gallstone disease in recurrent pancreatitis[23,24].

Sphincter of oddi dysfunction (SOD) is another common cause of ARP and is probably the most common cause of the idiopathic form. SOD comprises two clinical entities: (1) SO increased basal pressure, which refers to a structural alteration of the sphincter, as consequence of a long-lasting inflammatory process with subsequent fibrosis (stenosis); and (2) SO dyskinesia, referring to a transient primary motor abnormality characterized mainly by sphincter hypertone. Surgical specimens of the sphincter obtained from SOD patients show inflammation, muscular hypertrophy, and fibrosis of the Vater's ampulla in approximately 60% of patients while a primary motor disorder may occur in the remaining 40% of cases with normal histology[25]. SOD has been classified under three headings on the basis of clinical and morphological parameters[26] and may involve either the biliary or the pancreatic segment of the sphincter[27]. Type 1 dysfunction patients have acute pancreatitis (pancreatic-like pain with high serum pancreatic enzymes) together with a dilated common bile and/or main pancreatic duct and prolonged drainage, suggesting a structural abnormality (ampullary stenosis). Type II dysfunction patients have pancreatic-like pain, associated with one or two type I items; in this group, with either pancreatitis or only pancreatic-like pain patients with functional or structural sphincteric disorder are probably evenly distributed. Manometry shows elevated basal sphincter pressure but no stenosis in the majority of patients. Type III dysfunction patients have only pancreatic-like pain with no rise in serum pancreatic enzymes and bilio-pancreatic morphological abnormalities. By definition, type III SOD is not considered in case of recurrent pancreatitis.

SOD can affect either the biliary sphincter, pancreatic sphincter, or both. Judging from manometric findings from published data, SOD involves the biliary and/or pancreatic sphincter in respectively 65%-92% and 85%-100% of type I SOD, 58%-65% and 55%-67% of type II, and in 35%-59% and 28%-59% of type III. In a series by Eversman *et al*[27], among 123 patients labeled as type II, SOD was diagnosed in 65%; respectively 22%, 11%, and 32% had elevated basal sphincter pressure in the pancreatic sphincter only, biliary sphincter only, or both. However, normal basal pressure does not mean there is no fluctuating dysfunction or exclude a role of the sphincter in the recurrence of pancreatitis.

Other acquired anatomical conditions that may be associated with obstructive mechanisms are periampullary diverticula, benign and malignant tumors of the Vater's papilla or pancreatico-biliary junction, organic strictures of the main pancreatic duct, and cystic neoplasms, including mucinous ductal ectasia. Rare conditions associated with ARP are choledochocele and ampullary choledochal cysts.

There is still debate about whether periampullary diverticula are directly involved in the recurrence of pancreatitis; although these diverticula are frequently found in both gallstone and recurrent pancreatitis in middle-aged subjects, it has yet to be proved that they play any role in the occurrence of pancreatitis.

Organic strictures of the main pancreatic duct may be neoplastic or the consequence of a fibrotic process induced by a previous acute pancreatitis or pancreatic trauma, or a chronic disease. Neoplastic strictures are found to induce acute pancreatitis in about 5% of cases; among cystic neoplasms, mucinous ductal ectasia is the one most frequently associated with ARP or intermittent pancreatic-like pain.

Choledochocele is a congenital or acquired condition in which the intramural segment of the common bile duct is dilated and herniates into the duodenal lumen. Acute pancreatitis may develop when the cystic dilation or bile duct sludge or stones obstruct the pancreatic juice outflow. Endoscopically, the papilla shows a bulge into the duodenum, mainly involving the caruncula, and is soft when pressure is applied with the ERCP catheter. Ampullary choledochal cysts can develop when there is SOD.

Anatomical variants

Pancreas divisum is the most common variant of pancreatic ductal anatomy, occurring in up to 12% of individuals. Partial fusion of the ventral and dorsal ducts characterizes the incomplete (functional) pancreas divisum, in which the dorsal duct can drain through the major papilla *via* a communicating branch of the ventral duct. However, this communication is generally narrow and may be inadequate for draining the pancreatic secretion. The inability of minor papilla to accommodate the flow of pancreatic juice when the gland is stimulated leads to ductal hypertension that in some individuals may cause either recurrent pain shortly after a meal, or a persistent asymptomatic rise in serum pancreatic enzymes, or acute

relapsing pancreatitis. Persistent obstruction may lead to a chronic obstructive pancreatitis.

Although one retrospective series found no correlation between pancreas divisum and ARP[28], most studies show a significantly higher prevalence of this congenital variant in this patient population[29-33]. Dilation of the dorsal duct confirms the presence of some obstruction at the level of minor papilla and suggests a positive outcome after sphincterotomy or stenting.

Annular pancreas is a rare anatomical condition that may be associated with duodenal or biliary obstructive symptoms, as the consequence of the entrapment of both the duodenum and common bile duct by the annular growth of the gland[34-36]. About one third of patients with annular pancreas also have pancreas divisum, so it is not clear whether recurrent pancreatitis depends on the annular variant or on the pancreas divisum.

The presence of a common pancreatico-biliary channel abnormally long without sphincters separating the biliary and pancreatic ducts is a condition that facilitates free reflux of bile and pancreatic juice into the alternative duct. This abnormality of the pancreatico-biliary junction is easily diagnosed by MRCP or ERCP. Choledochal cysts are frequently associated with this kind of junction.

Other anatomical variants of the pancreatic ductal system and junction between the ventral and dorsal ducts may induce an impaired outflow of pancreatic juice into the duodenum and could explain the pancreatic pain and recurrent pancreatitis in some cases, when other causes have been excluded. The most frequent findings are a loop or sigmoid configuration of the main pancreatic duct.

Genetic causes

Genetic mutations have long been suspected as being associated with ARP and development of chronic pancreatitis over time[37]. Inherited conditions that can induce ARP are the cystic fibrosis transmembrane conductance regulator-gene (CFTR-gene), PRSS1-gene and SPINK1-gene mutations.

CFTR-gene mutations: This condition represents the most common inherited disease of the exocrine pancreas. Some phenotypic *CFTR*-gene mutations occur in about 5% of the Caucasian European and North American populations; however the true incidence of *CFTR*-gene mutations is probably underestimated[38-41]. Mutations of *CFTR*-gene induces a defect in chloride ion transport at the level of the apical membrane-chloride channels of epithelial cells, resulting in an abnormally viscous exocrine secretion that leads to persistently high intraductal pancreatic pressure. Over time, this chronic condition leads to secondary chronic obstructive ductal changes.

There are many clinical features associated with *CFTR*-gene mutation phenotype. Exocrine pancreatic insufficiency with no inflammatory changes is the most common finding; ARP may be the only clinical sign in some patients; asymptomatic persistent pancreatic hyper-

enzymemia with no morpho-functional pancreatic disorders may also be found. Subjects with non-functional CFTR protein show clinical features of cystic fibrosis. Those with less severe mutations in the *CFTR* gene risk developing pancreatitis, which is estimated to be 40 to 80 times that in the general population[42]. Heterozygotes for *CFTR* mutations are generally healthy but still have a 3 to 4-fold risk over the general population for pancreatitis.

PRSS1-gene mutations: Mutations in the cationic trypsinogen gene have been found in patients with hereditary pancreatitis[43,44]. In this autosomal dominant disorder the pancreas is unable to protect itself by premature or excessive trypsin activation in the gland; the lack of this protective mechanism against premature activation of trypsin predisposes individuals to recurrent bouts of pancreatitis in childhood and frequent progression to chronic pancreatitis.

Another pathogenic cofactor involved in ARP in presence of hereditary pancreatitis seems to be SOD; the dysfunction might be the consequence of the chronic inflammation of the sphincter induced by the passage of activated trypsin through it over time, in presence of a common biliopancreatic junction[45]. In these patients sphincterotomy may relieve symptoms, confirming a role of the high intraductal pressure induced by SOD or stenosis, but it does not significantly affect the progression of the acute recurrent disease to chronic pancreatitis. Patients with a history of first- or second-degree relatives with early-onset or recurrent episodes of acute pancreatitis of unknown etiology should be considered for genetic testing.

SPINK1-gene mutations: Another group of mutations that predispose to pancreatitis are in the serine protease inhibitor Kazal type I gene (*SPINK1*). *SPINK1* has a protective action in the pancreas since it serves as a critical feedback inhibitor of trypsin. Therefore, in a state of retained SPINK1 protein function due to a *SPINK1* gene mutation (mostly heterozygous), the pancreas is more likely to develop pancreatitis from other genetic or environmental factors. It has been repeatedly shown that 16%-23% of patients with apparent idiopathic pancreatitis have *SPINK1* mutations, compared with only about 2% of healthy controls[46]. These mutations have been estimated to raise the risk for pancreatitis about 12-fold over the general population.

Other causes

Well-known metabolic causes persisting over time that can induce ARP are hypertriglyceridemia and hypercalcemia. There are many causes of hypercalcemia, but the majority of patients who develop ARP have hyperparathyroidism. The diagnosis may be missed if calcium levels are not measured during each attack. Hypertriglyceridemia is increasingly common in Western countries in the setting of metabolic syndrome. Typically, serum triglycerides have to exceed 1000 mg/dL to precipitate an attack

of acute pancreatitis. Control of diabetes mellitus, weight loss, and lipid-lowering agents can reduce the triglyceride levels, but non-compliance is frequent and many of these patients progress toward chronic pancreatic damage.

Low serum levels of antioxidants, as selenium, vitamins A, C and E, and riboflavin, have been found in patients with chronic pancreatitis, probably because a deficient diet. The observation that selenium levels were lowest during acute bouts of pancreatitis[47] stimulated investigators to assess the antioxidant profile also in patients with ARP; however, the hypothesis that acute cellular injury determined by an uncontrolled free radical activity may be the cause of unexplained recurrent pancreatitis in some patients was not confirmed, as the antioxidant profiles were similar to those of control subjects[48]. Excess alcohol consumption is responsible for about 30% of all cases of acute pancreatitis in the United States[49]. Alcohol-induced acute pancreatitis typically occurs in people who have consumed large amounts of alcohol for at least 5-10 years. Recurrent episodes of acute alcoholic pancreatitis typically occur in patients with existing chronic pancreatitis[50]. Alcohol intake causes a transient stimulation of exocrine pancreatic secretion by increasing the synthesis and secretion of digestive and lysosomal enzymes in pancreatic acinar cells. Alcohol also sensitizes pancreatic acinar cells to cholecystokinin and may have a direct toxic effect on the acinar cells. However, these mechanisms alone are probably not sufficient to cause acute pancreatitis. Therefore, additional genetic and environmental factors are thought to influence the development of the disease.

Smoking has long been thought to play a role in the induction of acute pancreatitis, but it was only recently that large prospective studies have proved that cigarette smoking is an independent risk factor. The duration rather than the intensity of smoking increases the risk of non-gallstone-related acute pancreatitis. The risk of pancreatitis was reduced to a level comparable to that of non-smokers only two decades after smoking cessation[51]. However, data regarding the role of smoking in recurrent pancreatitis are lacking.

Many medications have been recognized as causes of acute pancreatitis, by a dose-dependent or hypersensitivity-related mechanism. For a number of substances there is general agreement on some relation with acute pancreatitis, and a recent review by the Midwest Multicenter Pancreatic Study Group[52] listed the following as medications for which a strong association with pancreatitis is documented by at least one positive rechallenge: alpha-methyldopa, 5-aminosalicylate, azathioprine, cimetidine, cytosine arabinoside, corticosteroids, estrogens, furosemide, isoniazid, mercaptopurine, metronidazole, pentamidine, procainamide, sulfamethazole, sulindac, tetracycline, trimethroprim/sulfamethoxazole and valproic acid.

DIAGNOSIS

It is extremely important to establish the cause of episodes of acute pancreatitis because by removing it we eliminate the risk of further recurrences if there is no chronic underlying disease involved. The patient's history and standard diagnostic tests such as blood chemistry, trans-abdominal ultrasound, MRCP, and CT scan generally detect the causes of recurrent episodes in about 70% of cases. When no cause is found at the initial diagnostic work-up, these patients should have a more advanced diagnostic work-up, that includes specific pancreatic tests, genetic testing, MRCP with secretin stimulation, sphincter of oddi motility evaluation, EUS, and in selected cases ERCP. Genetic and autoimmune pancreatitis can be diagnosed by testing respectively for *CFTR* or *SPINK1/PRSS1* gene mutations and IgG 4.

MRCP with the secretin test (MRCP-S) gives details of the morphology of the pancreatico-biliary ductal system and permits indirect evaluation of sphincter of oddi motility, as an alternative to more invasive tests such as manometry. However, the secretin test is less sensitive than manometry for intermittent sphincter motility disorders like types Ⅱ and Ⅲ SOD. Injection of secretin (1 IU/kg i.v. bolus) enhances the pancreatic ductal morphology, by stimulating pancreatic secretion of water and bicarbonates, and permits an evaluation of the kinetics of the main pancreatic duct (MPD), by measuring the duct diameter as an indirect indicator of pancreatic juice outflow through the papilla of Vater. The diameter of the MPD is measured at baseline at the body of the gland, then secretin is injected. The basal MPD diameter is considered normal when it is $\leqslant$ 3 mm; changes in MPD diameter (millimeters) are then measured at 1-min intervals for 15 min. The mean of the measurements at the last three one-minute intervals (from 13 to 15 min) is taken as the final value[53]. A Δ final-basal MPD caliber > 1.0 mm is considered diagnostic for some SOD[54,55].

Three studies employed MRCP-S for the diagnosis of SOD. One found no differences in normal subjects and SOD patients[55]; another found sensitivities of 37% and 62.5% and specificities of 85% and 85% in types Ⅱ and Ⅲ SOD[56]; the third found 57.1% sensitivity and 100% specificity in idiopathic pancreatitis[57].

Sphincter of oddi manometry (SOM) is still the gold standard for the diagnosis of SOD. SOM is performed during ERCP and requires selective cannulation of the bile duct and/or pancreatic duct through the major papilla with a triple-lumen, 5 F manometry catheter. Normal basal sphincter pressure usually does not exceed 35 mmHg (mean 15 mmHg); a basal pressure higher than 40 mmHg is considered abnormal. Abnormal basal sphincter pressure may be found in one or both sphincters. Raddawi and coll[58]. reported that abnormal basal sphincter pressure was mainly confined to the pancreatic duct segment in patients with ARP and to the bile duct segment in patients with biliary-type pain and abnormal liver function tests. In patients with SO stenosis manometric recording is reproducible and does not respond to muscle relaxants. In patients with SO dyskinesia there is a variety of abnormalities of wave propagation and/or frequency; the abnormal basal sphincter pressure or motility responds to muscle relaxants, and the sphincter may give a paradoxi-

cal response to i.v. cholecystokinin (CCK).

Indications for SOM have been developed according to the modified Hogan-Geenen SOD classification system. Type I SOD does not require manometric investigation for confirmation, since a structural disorder of the sphincter (stenosis) occurs in this situation. These patients have the best outcomes after biliary and/or pancreatic sphincterotomy.

In type II SOD with dilated ductal system, the basal sphincter pressure has been found abnormally elevated in the majority of cases[27,59,60] but a normal pressure profile does not exclude a transient dysfunction. Manometry, therefore, does not substantially improve the diagnosis, while exposing patients to an increased risk of post-procedural pancreatitis. In cases with non dilated ducts an objective diagnosis of dysfunction can be obtained only by manometric recording of the biliary and pancreatic segments of the sphincter. Unfortunately, the frequency of abnormal manometric recordings in these patients is low and varies widely, ranging in published series from 15% to 50% for biliary[27,59,60] and 35% to 49% for pancreatic-type SO dysfunction[27,61].

Although they have not been thoroughly studied, SOM results have been found to predict outcome from sphincterotomy in SOD patients, with the highest success rate in patients with type I SOD[62].

Pancreatic stent as a diagnostic test to achieve pain relief and predict the response to more definitive therapy (sphincter ablation), has been tried only limitedly. Our group has used pancreatic 5 F and 7 F stenting in some patients with recurrent pancreatitis and non-dilated ducts, with significant reductions in pancreatitis episodes; in these cases pancreatic sphincterotomy was successful[63]. Pancreatic stenting in patients with normal pancreatic ducts may cause ductal and parenchymal injury if the stent is left, even if for a short time.

Botulinum toxin (Botox) is a potent inhibitor of acetylcholine release from nerve endings. In a preliminary trial Botox injection into the SO halved the basal sphincter pressure, with an effect lasting four months, and gave symptom improvement[64]. The effect of Botox injection has been investigated only in type III patients with manometric evidence of SOD: Botox injection has been shown to predict the patients whose symptoms were most likely to improve with endoscopic sphincterotomy in 44%-80% of cases[65].

Although further studies are still needed, Botox may serve as a diagnostic trial for symptomatic patients with uncertain or not documented SOD, with responders undergoing permanent sphincter ablation. Unfortunately, the short-lasting effect of Botox limits its indication as a trial only to patients with symptoms occurring at intervals of not more than three months.

EUS has the highest sensitivity for detecting microlithiasis and sludge, either in the gallbladder or in the common bile duct[66], pancreatic tissue fibrosis, and small ductal changes of both the main pancreatic duct and side branches. The diagnosis of biliary sludge can be very

challenging, even with EUS, particularly after cholecystectomy. In some patients with recurrent pancreatitis and normal pancreas at CT scan and MRCP, EUS identifies ductal and parenchymal abnormalities, suggesting a diagnosis of chronic pancreatitis[67,68]. The frequency of the diagnosis of CP in patients with ARP, on the basis of EUS criteria, ranges from 10%-30%[69,70].

ERCP should be considered for diagnostic purposes only in selected cases of uncertain ductal morphology even at MRCP-S and should be followed by immediate biliary and/or pancreatic sphincterotomy. The procedure is associated with a 3%-5% complication rate that may reach 30% in cases with SOD. This additional work-up usually leads to the diagnosis of microlithiasis or bile sludge, SOD, pancreatic ductal abnormalities, either congenital or acquired, or anomalous pancreatico-biliary junction, early chronic pancreatitis, and genetic or auto-immune disorders. After a complete additional advanced work-up, the etiology remains unknown in no more than 10% of recurrent pancreatitis, which can then be defined as true idiopathic recurrent pancreatitis.

THERAPY

The efficacy of therapy in patients with a history of ARP depends on two main factors: whether or not the bouts of acute pancreatitis occur in a normal pancreas or in a setting of chronic pancreatitis, and whether or not a cause can be identified and removed.

Therapeutic approach to recurrent pancreatitis of biliary etiology (documented or suspected)

Laparoscopic cholecystectomy is curative when gallbladder stones or sludge are detected; however, the clinical benefit for sludge is less evident. If only sludge is present or suspected and cholecystectomy is not considered, UDCA (usually 12 mg/kg) is an acceptable alternative, if necessary combined with endoscopic biliary sphincterotomy. In these cases, long-term therapy with bile acids is required, since the drug works slowly. In previous studies[16,24,14] patients treated with UDCA had a significantly lower rate of recurrent pancreatitis (approximately 20% with therapy compared with 60% without). Unfortunately, no studies have made a head-to-head comparison of cholecystectomy, endoscopic biliary sphincterotomy, and bile acid therapy.

In patients who have already undergone cholecystectomy but present repeated attacks of pancreatitis with signs suggesting a biliary origin, even if no stones or sludge are detected, endoscopic biliary sphincterotomy is the procedure of choice. Common bile duct stones have been found in 4%-24% of patients up to 15 years after cholecystectomy. In cases with no evidence of stones, UDCA is likely to be ineffective because removing the gallbladder markedly reduces or completely eliminates the bile crystals and sludge; sludge may form in cases with SOD leading to persistent or transient bile flow obstruction. Endoscopic biliary sphincterotomy is the only effec-

tive treatment in these patients.

Therapeutic approach to recurrent pancreatitis associated with SOD

SOD is reported in about one third of cases with recurrent pancreatitis. It is still not clear whether biliary sludge or crystals, or inflammation cause sphincter malfunction, because of conflicting data and a lack of specific studies. The therapeutic approach in patients with SOD aims at reducing the resistance caused by the sphincter to the flow of bile and/or pancreatic juice. In documented SOD endoscopic sphincterotomy is currently the standard therapy. Non-invasive therapies, such as bile acids, calcium channel blockers, nitrates, and anticholinergic drugs, have proved unsuccessful in most cases and are not effective when an organic stricture involves the sphincter (type I dysfunction).

In patients with uncertain documentation of dysfunction the risks and benefits of endoscopic sphincterotomy should be carefully weighed before recommending it, because SOD patients have an ERCP-related complication rate that is markedly higher compared to patients with ductal stones.

Medical therapy of SOD has been investigated only on a small scale, by using drugs that relax smooth muscle. Sublingual nifedipine and nitrates have been found to reduce the basal sphincter pressure and achieve clinical benefit in up to 75% of patients. Drawbacks of medical therapy are the systemic side effects of the drugs, tachyphylaxis, and lack of long-term outcomes from regular therapy. Nevertheless, because of the "relative safety" of medical therapy, it should be considered in suspected type Ⅱ SOD with non-dilated ducts before considering more aggressive sphincter ablation.

Besides smooth muscle relaxants, other drugs can be used in these patients. Oral UDCA has been shown to be effective in patients with idiopathic recurrent pancreatitis, confirming the role of bile microlithiasis or sludge in SOD.

Endoscopic sphincterotomy may ablate either the biliary or the pancreatic segment of the SO, or both. In general, biliary sphincterotomy is done first and leads to clinical improvement in about 80% of cases; in case of failure, pancreatic sphincterotomy is done, preceded or not by further function testing. In some centers, biliary and pancreatic sphincterotomy are done at the same time, considering the high probability of a consensual sphincter dysfunction.

In SOD patients, endoscopic sphincterotomy is associated with a high rate of acute post-procedure pancreatitis (up to 20% of cases). To reduce such a risk, endoscopic techniques have been used (pancreatic duct stenting after biliary or pancreatic sphincterotomy and naso-pancreatic drainage after pancreatic sphincterotomy) to limit this complication.

Clinical improvement after sphincterotomy has been reported in 55%-95% of patients, depending on the type of SOD, according to the modified Hogan-Geenen classification system, and manometric recordings. Nineteen studies have been published, including up to 237 patients, with follow-up ranging from a mean of three months to five years[71,72]. Favorable outcomes are highest in type I SOD: in these patients improvement was reported in 83%-100% of cases. In type Ⅱ SOD patients, with functional sphincter disorder, long-term symptom relief was reported in up to 79%, depending on whether manometry was abnormal (best results) or normal. In patients without documented SO abnormalities, intrasphincteric botulin toxin injection could be considered before sphincterotomy and might help identify a transient SOD undetected by functional tests[73]. Since botulin toxin induces sphincter relaxation lasting no more than three months, however, it should be use to predict those most likely to benefit from endoscopic sphincterotomy, in patients with recurrent pancreatitis with normal morpho-functional findings[74]. This approach is adopted in only a few centers.

Compared to biliary sphincterotomy alone, dual sphincterotomy has been shown to have significantly better outcomes in the majority of studies, even if in a recent study dual sphincterotomy and biliary sphincterotomy had similar effects in preventing recurrence of acute pancreatitis[75].

Endoscopic sphincterotomy may fail to achieve symptom relief in documented SOD in the following conditions: (1) biliary sphincterotomy has been inadequate or re-stenosis has occurred. Sphincterotomy should be revised; if no "cutting space" remains balloon dilation should be considered; (2) pancreatic sphincter has residual abnormal basal pressure. A persistent elevated basal pressure of the pancreatic sphincter has been reported in approximately 90% of patients with persistent pain or pancreatitis after biliary sphincterotomy. Endoscopic pancreatic sphincterotomy achieves symptomatic improvement in 60%-90% of these patients. As an alternative, if pancreatic sphincterotomy has not been done, an empirical therapeutic approach may be attempted by placing a small-caliber (5-7 F) pancreatic stent; the stent may be effective and helps to predict whether there is any persisting outflow obstruction. However, in a normal pancreas pancreatic stenting induces chronic pancreatitis-like ductal changes and should therefore be done only for a short period (no longer than three months); moreover, few published data on pancreatic stenting for SOD are available and most of them so far are disappointing; and (3) patients may fail to respond to sphincterotomy because they have chronic pancreatitis, even with an apparently normal pancreatogram. In these patients EUS may show parenchymal and ductal changes suggesting early-stage chronic pancreatitis.

The surgical approach most commonly used is transduodenal biliary sphincteroplasty with trans-ampullary septoplasty. Outcomes are similar to those of endoscopic sphincterotomy while complication rate and cost of care are higher, so the surgical approach for SOD has largely been replaced by endoscopic therapy.

Therapeutic approach to recurrent pancreatitis associated with pancreas divisum

Pancreas divisum is reported in about 20% of patients with ARP. Endoscopic and surgical therapy are comparably effective in 70%-90%[76] so endoscopic therapy is preferred in most cases. It is still not clear whether endotherapy should be considered only when there is a dilated dorsal duct and whether it can prevent the risk of progression toward chronic pancreatitis. In cases with a non-dilated dorsal duct, the MRCP-S test may help detect some minor papilla malfunction and select the therapy.

Endoscopic therapy includes minor papilla sphincterotomy or stenting, or catheter dilation. In patients with dilated dorsal duct or abnormal function test, and no ductal strictures upstream of the minor papilla, sphincterotomy is the procedure of choice. After sphincterotomy, a short-term dorsal pancreatic duct stent placement is recommended to avoid post-procedure strictures and procedure-related complications. In cases without dorsal duct dilation or abnormalities and with a normal function test, dorsal pancreatic duct short-term stenting should be considered, to identify patients who can benefit from minor papilla sphincterotomy.

If pancreatitis still recurs after sphincterotomy, pancreatic stenting may be useful, with 7 F to 10 F stents depending on the dorsal duct dilation. If ductal strictures are documented, catheter dilation followed by stenting is the therapy of choice.

Although endoscopic therapy has been proved effective in a large percentage of cases, only one randomized controlled trial examined a small number of cases: in the treatment group, 9 out of 10 patients (90%) had no further episodes of acute pancreatitis during a three-year follow-up, while 6 of 9 patients (67%) who were randomized to no treatment had at least one episode[77]. A still unsettled issue is whether a tendency towards chronic pancreatitis persists in pancreas divisum patients, even after successful treatment, and if so, why.

Therapeutic approach to recurrent pancreatitis associated with other lesions obstructing the flow of pancreatic juice

Any process preventing the free flow of pancreatic juice can lead to ARP. The lesions can be at the level of Vater's papilla or around it, or in the pancreatico-biliary ductal system. Treatment aims to relieve the obstruction and re-establish the free flow.

Ampullary adenomas and carcinomas are the commonest causes of papillary lesions and can be resected either surgically or endoscopically. Ampullary tumors generally have a more favorable outcome than pancreatic tumors. Independently from the histological diagnosis, EUS should be done to establish whether an endoscopic or surgical approach is most likely to be curative. When the lesions are confined within the muscolaris mucosae and do not involve the biliary or pancreatic duct, endoscopic resection should be preferred. The tumor can be excised en-bloc or piecemeal by snare ampullectomy. En-

bloc resection should be done in cases with a lesion confined within the ampulla; in these cases there is no need to lift the mucosa by submucosal injection of saline solution, glycerol or hyaluronic acid, because this maneuver may make it more difficult to resect the lesion completely. Piecemeal resection is done when the lesion tends to extend around the papilla; in these conditions, lifting the mucosa from the submucosa permits complete and safer resection of the adenomatous tissue.

In patients with an abnormally long (> 15 mm) common pancreatico-biliary channel the sphincter does not separate the bile and pancreatic ducts so bile can flow into the pancreatic ductal system and lead to pancreatitis. Endoscopic biliary sphincter ablation avoids the bile flow into the pancreatic duct and reduces the intra-ampullary resistance to pancreatic juice flow, thus reducing the risk of recurrences of pancreatitis.

To deal with choledochocele endoscopic section by biliary sphincterotomy or needle-knife is usually effective, though a few patients require surgical sphincteroplasty. The surgical approach should be preferred for large lesions.

Segmental strictures of the main pancreatic duct are found in 5%-10% of patients with ARP[78] and may be related to chronic pancreatitis, residual scars in acute severe pancreatitis, pancreatic trauma, or neoplastic conditions. The differential diagnosis between the benign and malignant nature of the stricture is pivotal before planning treatment, which in most cases is endoscopic. EUS has swiftly became the preferred diagnostic procedure, because it offers the best sensitivity for identifying a pancreatic neoplasm for lesions 2-3 cm in diameter, and the diagnosis can be confirmed by EUS-guided fine-needle aspiration. ERCP should be done to confirm the diagnosis only in selected cases, by guide wire-guided intra-ductal brush cytology or, more recently, confocal endomicroscopy.

Endoscopic treatment should be done with curative purpose for benign lesions and palliation for malignant lesions unsuitable for curative surgery, and consists of ERCP-guided stricture dilation and stenting. Stenting of benign strictures will be planned for one or -better -two years, with three-monthly stent exchange, using progressively larger stents, to achieve lasting dilation and resolution of symptoms.

Cystic pancreatic tumors may also be associated with ARP. Serous cystadenomas are benign and can be managed conservatively. Mucinous tumors (cystadenomas and adenocarcinoma, or intraductal papillary mucinous neoplasia-IPMN) are pre-malignant or malignant, and require follow-up and surgical resection when there are worrisome features or high-risk stigmata. For IPMN involving the main pancreatic duct the risk of malignant evolution is documented, but the data are still uncertain for IPMN involving side branches. IPMN involving the main pancreatic duct more frequently causes recurrent pancreatitis, since the abnormal mucin secretion produces a dense pancreatic juice that leads to intraductal hyperten-

sion. Endoscopic biliary and pancreatic sphincterotomy facilitates the juice outflow through the papilla and may help prevent episodes of acute pancreatitis in patients in a follow-up program.

Annular pancreas is another congenital abnormality of the pancreatic ductal system seen in patients with recurrent pancreatitis. Surgical resection of the lesion is the treatment of choice in these cases.

Recurrent pancreatitis associated with the *CFTR*-gene mutation of hereditary pancreatitis may be prevented by endoscopic pancreatic sphincterotomy in selected cases with a dilated pancreatic duct; this procedure facilitates the outflow of pancreatic juice, which is particularly dense in patients with *CFTR*-gene mutations and may cause intraductal hypertension. However, as yet there are no prospective studies demonstrating that decompressive therapy can favorably alter the course of the disease.

In conclusion, a careful diagnostic algorithm serves to identify the etiology of ARP in up to 90% of cases, while in the remaining cases the cause remains unknown. The introduction into clinical practice, besides CT scans and ERCP, of genetic testing, SO manometry, MRCP and EUS with secretin, and botulin toxin injection, have markedly improved the diagnostic yield. Because occult bile stone disease and SOD account for the majority of cases, cholecystectomy, and if necessary endoscopic biliary and/or pancreatic sphincterotomy are curative in most cases of ARP. Endoscopic biliary sphincterotomy appears to be curative *per se* in about 80% of patients. Oral UDCA alone has also been reported effective for the treatment of biliary sludge and possible related SOD in some studies. In uncertain cases botulin toxin injection may help identify some cases of SOD, but this treatment is not widely used.

In the last twenty years, pancreatic endotherapy has been proved effective in cases of recurrent pancreatitis depending on pancreatic ductal obstruction, independently from the cause of obstruction, and has been widely used instead of more aggressive approaches. However, there is as yet no long-term follow-up to assess the progression of the disease, independently of the therapeutic success.

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2014 ADVANCES IN PANCREATITIS

Hormonal protection in acute pancreatitis by ghrelin, leptin and melatonin

Jolanta Jaworek, Stanisław Jan Konturek

Jolanta Jaworek, Department of Medical Physiology, Faculty of Health Sciences, Jagiellonian University Collegium Medicum, 31-125 Kraków, Poland

Stanisław Jan Konturek, Chair of Physiology, Medical Faculty Jagiellonian University Collegim Medicum, 31-125 Kraków, Poland

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Correspondence to: Jolanta Jaworek, MD, PhD, Department of Medical Physiology, Faculty of Health Sciences, Jagiellonian University Collegium Medicum, Michałowskiego str. 12, 31-125 Kraków, Poland. mpjawore@cyf-kr.edu.pl

Telephone: +48-12-6343397 Fax: +48-12-6343397

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Abstract

Acute pancreatitis is a nonbacterial disease of the pancreas. The severe form of this ailment is characterized by high mortality. Whether acute pancreatitis develops as the severe type or resolves depends on the intensity of the inflammatory process which is counteracted by the recruitment of innate defense mechanisms. It has been shown that the hormones ghrelin, leptin and melatonin are able to modulate the immune function of the organism and to protect the pancreas against inflammatory damage. Experimental studies have demonstrated that the application of these substances prior to the induction of acute pancreatitis significantly attenuated the intensity of the inflammation and reduced pancreatic tissue damage. The pancreatic protective mechanisms of the above hormones have been related to the mobilization of non-specific immune defense, to the inhibition of nuclear factor kappa B and modulation of cytokine production, to the stimulation of heat shock proteins and changes of apoptotic processes in the acinar cells, as well as to the activation of antioxidant system of the pancreatic tissue. The protective effect of ghrelin seems to be indirect and perhaps dependent on the release of growth hormone and insulin-like growth factor 1. Leptin and ghrelin, but not melatonin, employ sensory nerves in their beneficial action on acute pancreatitis. It is very likely that ghrelin, leptin and melatonin could be implicated in the natural protection of the pancreatic gland against inflammatory damage because the blood levels of these substances increase in the initial phase of pancreatic inflammation. The above hormones could be a part of the innate resistance system which might remove noxious factors and could suppress or attenuate the inflammatory process in the pancreas.

Core tip: The pathogenesis of acute pancreatitis is not clear and treatment of this disease is unspecific. Since the severe form of acute pancreatitis often leads to death or to pancreatic insufficiency, the understanding of the mechanisms involved in pancreatic protection appears to be an important problem. Experimental data have shown that pancreatitis severity could be attenuated by various hormones. Herein, we review the results of our research studies and others, as well as data from clinical observations concerning the protective effects of ghrelin, leptin and melatonin on acute pancreatitis. We also present the hypothetical mechanisms responsible for the beneficial influence of these substances on pancreatic inflammation.

INTRODUCTION

Acute pancreatitis is a sterile inflammatory disease of the pancreatic tissue. This ailment has been classified as mild, moderate or severe[1]. Mild pancreatitis is the most common, local pancreatic inflammation which resolves by itself. Moderate or severe acute pancreatitis with organ failure requires prolonged and expensive hospitalization, is characterized by high mortality and often leads to endocrine or exocrine dysfunction of the pancreas[2,3]. Despite the intensive research and clinical studies of the last decades, the pathogenesis of acute pancreatitis still remains unclear and the treatment of this disease is not specific. It is believed that the key determinant of this disease is the intra-acinar activation of digestive enzymes (mainly trypsin) and autodigestion of the pancreatic gland which initiates local (mild pancreatitis) or systemic (moderate and severe form of this disease) inflammation[4].

The cellular mechanisms responsible for zymogen activation are not fully understood. One possible explanation could be an impaired autophagy process resulting from lysosomal dysfunction and imbalance between two lysosomal enzymes: cathepsin B, which cleaves trypsinogen into active trypsin, and cathepsin L, responsible for trypsin degradation. The abnormal function of mitochondria and ATP depletion suppresses apoptosis and promotes acinar necrosis[5]. Damaged acinar cells release toxic substances, known as damage associated molecular patterns (DAMP), to the extracellular space leading to the propagation of the sterile inflammatory process[6]. Another hypothesis shows that activation of the nuclear NFκB, observed in the early step of acute pancreatitis, could be related to the cleavage of trypsinogen into active trypsin. However, the relationship between these phenomena has not been elucidated[7]. Recently, an interesting theory concerning the role of the pancreatic duct cells in the pathogenesis of acute pancreatitis has been presented. It has been explained that the reduction of bicarbonate secretion by duct cells leads to the acidification of the luminal space and contributes to the intracellular zymogen activation[8].

The inflammatory process in the pancreas is counteracted by activation of innate defense systems which could remove injurious agents to suppress or reduce pancreatic inflammation. Stimulation of the immune cells, production of protective substances and anti-inflammatory cytokines, modulation of the apoptotic signaling pathway and activation of antioxidant defense systems are among the mechanisms implicated in the cellular and tissue resistance[9-12]. Identification of the factors which trigger the defense systems against acute pancreatitis could be of great importance for the creation of new therapeutic strategies in this disease.

Previous experimental studies on acute pancreatitis have evidenced that non-specific immunity and innate defense systems could be activated in several ways and by various factors, such as stimulation of nitric oxide synthase (NOS) and generation of nitric oxide (NO),

thermal preconditioning and administration of low doses of biologically active substances or endotoxins[12-14]. Some hormones have been shown to protect the pancreas against the development of acute pancreatitis and to attenuate the course of this disease. Suppression of pancreatic inflammation and a marked reduction of tissue inflammatory damage have been observed as the result of the application of the hormones ghrelin, leptin and melatonin.

GHRELIN

Structure, tissue expression, receptors and biological effects

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, was originally isolated from the rat stomach by Kojima *et al*[15]. The main source of ghrelin in the organisms of rats and dogs appears to be X/A cells of the oxyntic mucosa, which represent 20% of the mucosal cell population, whereas in humans, ghrelin has been found in the P/D1 cell type[15,16]. Besides the stomach, ghrelin has been widely expressed in the additional tissues of the gastrointestinal tract (duodenum, jejunum, colon, pancreas), in the central nervous system (hypothalamus, cortex, brain stem, pituitary) and in other organs (kidney, heart, lung, testis, immune cells)[16-20]. In the pancreas, ghrelin-producing cells represent an independent cell population of the pancreatic islets and ghrelin is possibly involved in glucose homeostasis[21]. The ghrelin system (ghrelin and its receptor) also exists in the pancreatic acinar cells[22]. An experimental study has shown that ghrelin could be implicated in the stimulation of pancreatic enzyme secretion[23].

Part of ghrelin peptides undergoes posttranslational acylation, dependent on the enzyme ghrelin-O-acyltransferase (GOAT), and circulating ghrelin consists of two forms: desacyl ghrelin (90%) and acyl ghrelin (10%)[24]. Both forms of ghrelin are agonists of the ghrelin receptor; however, under physiological conditions, only acylated ghrelin is able to activate the intracellular signaling cascade of this receptor[15,25]. The desacyl ghrelin has previously been believed to be a degradation product of acyl ghrelin but recent observations have shown that desacyl ghrelin is able to produce biological effects independent of those of acylated peptide. It has been reported that desacyl ghrelin prevented the activation of apoptosis and fibrosis of cardiomyocytes and protected endothelial cells from apoptosis induced by oxidative stress[26,27]. Administration of desacyl ghrelin to rats modulated body temperature and produced arterial dilatation, probably *via* activation of the parasympathetic nervous system[28]. On the other hand, desacyl ghrelin could antagonize the activation of the acylated form. All these observations suggest that desacyl ghrelin might be a separate hormone which interacts with its own specific, as yet unknown, receptor[29].

The ghrelin receptor (GHS-Rs) is a G-protein coupled

receptor characterized by transmembrane domains[23]. GHS-R has been identified as two spliced variants: functional ghrelin receptor type 1 (GHS-R1a) and non-functional, unspliced GHS-R1b. Both GHS-R1s have been predominantly expressed in the pituitary but this receptor has also been detected in the pancreas, spleen, heart, adrenal and thyroid glands[30,31].

Ghrelin has been shown to produce a wide range of biological effects in the organism, such as: (1) release of prolactin, adrenocorticotropic and growth hormones; (2) control of appetite and food intake; (3) stimulation of gastric and pancreatic secretion and gastrointestinal motility; (4) modulation of cardiovascular and reproductive functions; (5) increase of neoglucogenesis and adipogenesis; (6) stimulation of bone formation; and (7) modulation of immune functions[32]. Nevertheless, the physiological involvement of ghrelin in most of above functions is not completely clear.

Anti-inflammatory effects of ghrelin

The anti-inflammatory effects of ghrelin have been shown in many tissues, including the pancreas[33]. Ghrelin protects gastric mucosa against acute ulceration and accelerates the healing of chronic gastric and duodenal ulcers[34]. Ghrelin suppresses inflammation in sepsis and inflammatory bowel disease, reduces inflammatory pain and attenuated chronic liver injury[35-38]. The presence of ghrelin receptors on human peripheral lymphocytes, neutrophils and on the leukemic T, B and myeloid cell lines indicates that ghrelin is able to directly affect the functions of immune cells[20]. Indeed, ghrelin could inhibit production of anti-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 8 (IL-8)[33,38-40]. It has been demonstrated that the downregulation of pro-inflammatory cytokines by ghrelin is mediated by MAPK phosphatase-1 enzyme involved in the innate immune response[41]. In addition, ghrelin has been found to reduce the phagocytic activity of macrophages *in vivo* and *in vitro* and to decrease the production of high mobility box 1 protein (HMGB1)[42,43]. The anti-inflammatory effect of ghrelin could also be attributed to the activation of NOS and to enhanced production and release of NO[38]. Controversial reports have been presented concerning the effect of ghrelin on nuclear factor kappa B (NF-κB). In human B cells, ghrelin promotes NF-κB, whereas in the pancreas, ghrelin inhibits activation of this substance[44,45].

Ghrelin and acute pancreatitis

Numerous studies have shown that administration of ghrelin to animals prior to the induction of acute pancreatitis protected pancreatic tissue against damage and attenuated the inflammation[10,30,45-47]. Ghrelin reduced the morphological signs of acute pancreatic inflammation, diminished blood levels of IL-1β, decreased plasma lipase and improved DNA synthesis in rats subjected to caerulein-induced pancreatitis; however, pancreatic blood flow in these animals was unaffected by ghrelin[30].

The favorable effect of ghrelin on the pancreas has been also demonstrated in acute necrotizing L-arginine pancreatitis as well as in taurocholate-induced pancreatitis and is attributed to the inhibition of NFκ-B expression and the blockade of the inflammatory signal transduction pathway[44,45]. In addition, ghrelin has been demonstrated to lessen pancreatitis-associated lung injury, to reduce sequestration of neutrophils in the lung, to limit production of the proinflammatory cytokines, such as IL-6, and TNFα, and to inhibit pulmonary substance P expression[47,48].

Ghrelin could exert its positive effect on the pancreas *via* the central mechanism because the pancreatic protective effect of ghrelin was observed following application of this peptide into the cerebral ventricles of rats subjected to caerulein-induced pancreatitis. This protection was correlated with the release of growth hormone (GH) and was completely reversed by deactivation of sensory nerves with capsaicin[10]. Hypophysectomy cancelled out the pancreatic protection evoked by ghrelin and eliminated GH and insulin-like growth factor 1 (IGF-1) from the serum[46]. The above results present the evidence that the protective effect of ghrelin on the pancreas is indirect and depends on the activation of sensory nerves and the release of GH and IGF-1.

A recent report has shown the therapeutic effect of ghrelin in the course of caerulein-induced pancreatitis. Administration of ghrelin after the development of acute pancreatitis reduced the intensity of pancreatic inflammation and accelerated the regeneration of the gland. This effect was mainly related to the reduction of IL1β and to the stimulation of pancreatic cell proliferation[49].

It was demonstrated that in animals with acute pancreatitis, serum levels of endogenous ghrelin at 24 and 48 h were significantly increased compared to the control[47]. Clinical studies have shown that ghrelin levels measured at the first, third and fifth day of hospitalization in patients with acute pancreatitis were higher than in healthy individuals[50]. It was also reported that serum ghrelin levels were markedly elevated in patients with high risk factors for severe forms of acute pancreatitis[51]. It is very likely that ghrelin is released in high amounts in response to the initiation of the inflammatory process in the pancreas to suppress the inflammation and protect the pancreatic tissue.

All the above observations indicate that ghrelin could be implicated in the natural protection of the pancreatic tissue through the activation of the innate immune system to prevent the development of the inflammatory process in the pancreas. The pancreatic protective effect of ghrelin appears to be indirect and depends on the release of GH and IGF-1 by ghrelin (Figure 1).

LEPTIN

Structure, tissue expression, receptors and biological effects

Leptin is one of the adipokines, cytokine-like hormones
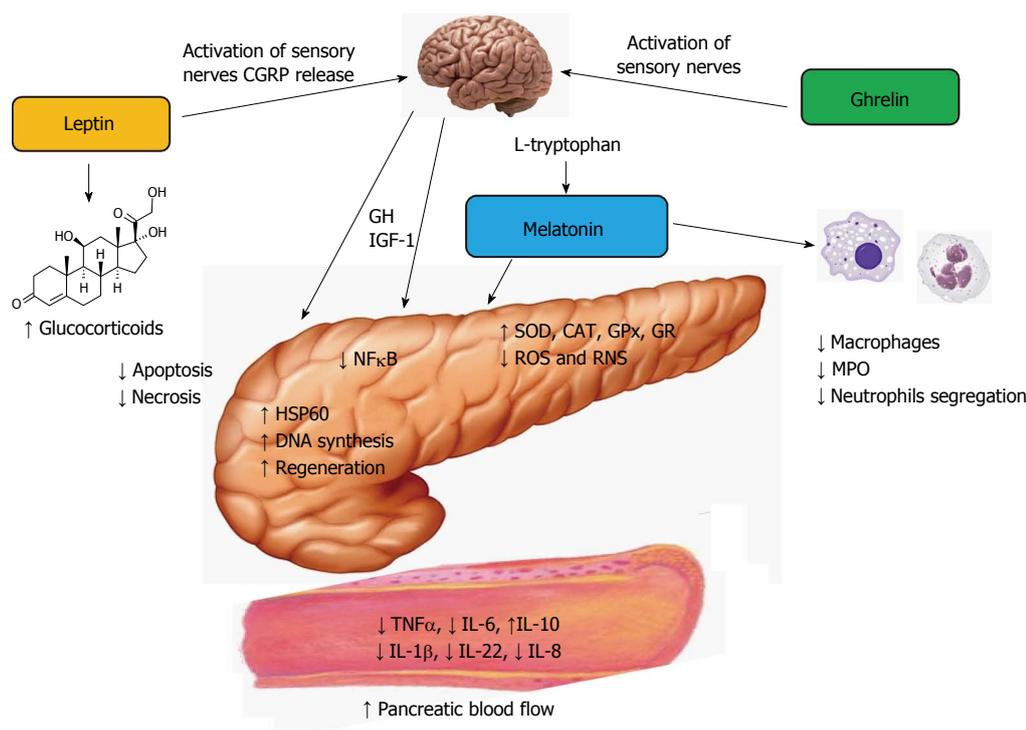
Figure 1 Hypothetical mechanisms of the protective action of ghrelin, leptin and melatonin on acute pancreatitis. GH: Growth hormone; IGF-1: Insulin-like growth factor; NF-κB: Nuclear factor kappa B; HSP60: Heat shock protein 60; nNOS: Neural nitric oxide synthase; NO: Nitric oxide; TNFα: Tumor necrosis factor α; IL: Interleukin; MPO: Myeloperoxidase; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; ROS: Reactive oxygen species; RNS: Reactive nitrogen species.

which are produced in white adipose tissue. Besides adipocytes, leptin has been also detected in other organs, such as the stomach, muscles and bones[52,53]. This 16-kDa protein is encoded by the *ob* gene and was first recognized as an inhibitor of appetite, a stimulator of energy expenditure and a regulator of body weight[54]. Leptin is well known as one of the main modulators of energy balance but is also implicated in the regulation of neuroendocrine and secretory functions of the body. Indeed, leptin has been shown to affect gastric and pancreatic secretions, insulin release, to protect the gastric mucosa against noxious agents and to influence the inflammatory process in the pancreas[55,56]. Leptin could be also involved in the modulation of several processes of the body, such as reproduction, angiogenesis and bone metabolism, but recent studies have focused on the role of leptin in immunity[57-59].

Leptin exerts its effect through its specific receptor (Ob-R) which belongs to the class I of the cytokine family, together with receptors for IL-6 and IL-12. Ob-R is represented by six isoforms of different length but only full-length Ob-Rb is able to activate the signal transduction pathway[60]. Ob-R is present in the hypothalamus, pituitary, kidney, lung, bone marrow, enterocytes, reproductive system and pancreatic acinar cells, as well as all types of immune cells[24,60,61].

Effects of leptin on immune functions

Leptin received particular attention as the main modulator of immune functions and inflammatory processes in the organism[59]. The reports concerning the effect of leptin on the immune processes are contradictory. In some studies, leptin has been shown to trigger inflammatory responses and to play an important role in chronic inflammation[62,63]. Other studies documented the anti-inflammatory effects of leptin[64,65].

It has been reported that leptin improved the function of macrophages, modulates lymphocyte proliferation, increases the ratio of T naïve/T memory cells and stimulates the production of pro-inflammatory cytokines, such as TNFα, IL-1 and IL-6[66]. Leptin production increases in acute inflammation and sepsis. It was proposed that leptin blood levels could be a predictive factor of the increased survival and attenuation of inflammation[67]. Additional evidence for the beneficial role of leptin in sepsis comes from the observation that the administration of exogenous leptin increased survival in septic mice[68].

Leptin and acute pancreatitis

Our previous reports have demonstrated that the application of leptin to rats prior to the induction of acute caerulein-induced pancreatitis significantly reduced the severity of pancreatic inflammation[69]. This beneficial effect of leptin has been observed following intraperitoneal as well as intracerebroventricular administration of this adipokine. The effect of leptin on acute pancreatitis was completely abolished by the deactivation of sensory nerves with capsaicin. These nerves employed calcitonin gene-related peptide (CGRP) as one of the neuromediators[70]. The pharmacological blockade of sensory nerves

with CGRP 8-37, an antagonist of CGRP, resulted in the total reversion of the favorable effects of leptin on acute pancreatitis[69]. This observation indicates that sensory nerves and CGRP play a part in the protection of the pancreatic gland afforded by leptin against acute damage. Subsequent studies have shown that leptin treatment ameliorates lung injuries in rats with the caerulein model of acute pancreatitis[71]. In addition, treatment with leptin accelerated pancreatic tissue repair in animals subjected to ischemic pancreatitis[72]. The positive effects of this adipokine on acute pancreatitis have been also observed in the model of acute pancreatitis evoked by ischemia/reperfusion and documented by histological assessment and significant reduction of TNFα and Il-1β blood levels[71,72]. The beneficial effect of leptin on acute pancreatitis could be attributed to the activation of the NOS/NO system and release of NO in the pancreas, to the improvement of pancreatic microcirculation and, at least in part, to the release of glucocorticoids which provide an unspecific attenuation of the inflammatory processes[61,62,69,72] (Figure 1). A study on the pancreatic acinar cell line AR42J revealed that leptin is able to increase the gene expression of heat shock protein 60 (HSP60) in these cells[73]. HSP60 belongs to the group of chaperone proteins, stimulated by high temperature, oxidative stress or inflammation. Activation of HSP60 by leptin could limit mitochondrial and nuclear injury, might prevent the endoplasmic reticulum from the damage and could reduce formation of autophagosomes[73,74].

More evidence concerning the important role of leptin in acute pancreatitis comes from experiments on congenitally obese mice with a deficient leptin system. In this study, obese knockout mice with deficits of leptin (Lep[Ob] mice) or leptin receptor (Lep[Db] mice) developed acute pancreatitis in the more severe form than the acute pancreatitis demonstrated in wild-type animals[75]. This observation clearly shows that the leptin system is implicated in the innate immune defense against acute pancreatic inflammation, yet leptin is not a sole player in the modulation of pancreatic resistance. Other adipokines, such as adiponectin, visfatin and resistin, could also play a role in the modulation of acute pancreatitis severity[59,76].

In contrast to the reports presenting the anti-inflammatory effects of leptin, recent publications have shown that leptin up-regulated the expression of toll-like receptor 2 (TLR-2) on human monocytes and increased TNFα expression stimulated by endotoxins. This activity of leptin may potentiate innate immunity and inflammation in obese hyperleptinemic patients[77]. Studies on obese (hyperleptinemic) rats with acute necrotizing pancreatitis indicated that in these animals the expression of pro-inflammatory IL-6 was higher, whereas the signal of anti-inflammatory IL-10 was lower than in the group of lean animals[78]. Some authors concluded that obesity and hyperleptinemia could be a risk factor for severe acute pancreatitis[76,79].

Changes of leptin level in acute pancreatitis have been the subject of numerous experimental and clinical studies. Many investigators have noted the considerable increases of leptin blood levels in acute pancreatitis in rats and humans[47,64,75,80]. Nevertheless, the leptin blood levels did not correlate with pancreatitis severity[47,80-82]. The recent comprehensive review of Karpavicius *et al*[83] analyzed the prognostic value of leptin and other adipokines in predicting the course of acute pancreatitis. The authors concluded that leptin levels increase in acute pancreatitis; however, these levels are not in parallel with the severity of acute pancreatitis and could not be used as a prognostic value of the course and possible complications of this disease[83].

The results of the above studies lead to the conclusion that leptin could be considered a factor which potentiates non-specific immune defense and stimulates inflammatory reaction. It has been shown that leptin blood levels increase in acute pancreatitis, yet these levels have not been related to the severity of this disease. It is very likely that leptin could activate the initial phase of the innate immune response and thus trigger the immune defense in the early stages of inflammation. Such a mobilization of the immune system might suppress the acute inflammation near the beginning and prevent the development of serious disease. Nevertheless, with the development of intensive inflammation, leptin could continuously activate the immune cells and interleukin production to combat the pathogens or factors responsible for inflammation. Constant stimulation of the immune system by leptin might favor the progression of an inflammatory state.

It is worth remembering that other adipokines besides leptin also participate in the modulation of inflammatory process and an imbalance between leptin and other adipokines could deregulate the immune response.

MELATONIN

Structure, tissue expression and receptors

Melatonin (5-methoxy-N-acetyltryptamine) is an indoleamine, produced from amino acid L-tryptophan in the four steps reaction with serotonin as a direct melatonin precursor[84]. This indoleamine is released from the pineal gland in the regular nocturnal/diurnal rhythm, with the peak at night[85]. Melatonin was discovered first in the pineal gland and is best known as the pineal hormone but this substance has also been found in numerous mammalian tissues, such as the retina, Harderian gland, brain and in the gastrointestinal system, which appears to be the richest source of melatonin in the organism[86-88]. Gastrointestinal melatonin originates from the enteroendocrine cells of the gut mucosa, from food ingested and from bile[87,89,90]. Food stimulates the release of melatonin in a manner independent of the light/dark cycle[88,91].

Melatonin could exert its biological effect *via* its specific receptors MT1, MT2 and MT3 on the cell membranes, but it could also directly enter into the cell because of its high solubility in lipids[92]. Melatonin membrane receptors MT1 and MT2 belong to 7-transmembrane G-protein-

coupled receptors and both could be antagonized by luzindole[93]. Melatonin receptor MT₃ is an enzyme quinone reductase 2 known as a potent antioxidant[93,94]. Melatonin could perhaps activate nuclear orphan receptors RZR/ROR but the role of these receptors is not known[94].

Melatonin receptors have been detected in many tissues, such as the central nervous system, cardiovascular system, immune cells, retina and the gastrointestinal system[94,95]. Both melatonin and its receptors have been identified in the human pancreatic tissue in the islet of Langerhans and this indoleamine has been suggested as one of the modulators of insulin secretion[95].

Anti-inflammatory effects of melatonin

Melatonin has been reported to produce a wide range of biological effects but its physiological role in the organism is still unknown. This substance was first recognized as part of the biological clock because of its rhythmic diurnal/nocturnal secretions[84,96]. Subsequent studies have shown a very special role of melatonin as a vitally important tissue protector and modulator of immune response. Melatonin enhances tissue defense against oxidative damage because it is a potent activator of antioxidant enzymes and a direct scavenger of radical oxygen (ROS) and nitrogen (RNS) species. ROS and RNS are produced in mitochondria and under normal conditions they are neutralized by antioxidant enzymes as well as by natural non-enzymatic scavengers, such as melatonin, glutathione, vitamins C and E and others[97,98]. Oxidative stress and inflammation resulted in the massive production of these free radicals which could not be counteracted by antioxidant defense of tissue, leading to tissue damage[99,100]. Melatonin also activates the second line of tissue defense which is dependent on the antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or glutathione reductase (GR)[101-103].

Besides its antioxidant properties, melatonin could strengthen the immune defense through the modulation of the signal transduction pathways involved in inflammatory processes. Melatonin inhibits the nuclear binding of NF-kappaB and in this manner it could reduce the production of pro-inflammatory cytokines (TNFα and interleukins IL-1β, IL-6, IL-8)[104]. This indoleamine has been reported to reduce prostaglandin synthesis, to modulate cell apoptosis and necrosis, and to affect the process of angiogenesis[105-107].

Melatonin and acute pancreatitis

Experimental studies have shown that the administration of melatonin to rats prior to the induction of acute pancreatitis protected the pancreas from the development of acute inflammation and significantly diminished pancreatic tissue damage[108-114]. Melatonin radically reduced the morphological signs of inflammation, such as edema, leukocyte infiltration and the vacuolization of the acinar cells[10,111,115-117]. The blood levels of amylase and lipase, the indicator enzymes of acute pancreatitis severity, as well as blood levels of pro-inflammatory cy-

tokine TNFα, were significantly reduced in the animals pretreated with melatonin and subjected to acute pancreatitis[10,106,110,113,115-118]. In contrast, the blood level of anti-inflammatory IL-10 was increased markedly in these animals[10,116].

It is worth noting that the favorable effects of melatonin on acute pancreatitis were paralleled by its precursor, L-tryptophan. Application of L-tryptophan to animals produced the significant and dose-dependent increase of melatonin blood level. This observation indicates that the anti-inflammatory effects of L-tryptophan are probably dependent on the conversion of this amino acid to melatonin. The above favorable effects of melatonin or L-tryptophan have been observed in rats subjected to caerulein-induced pancreatitis as well as in those with pancreatitis induced by ischemia-reperfusion[108]. Nevertheless, the beneficial effects of melatonin on acute pancreatitis have been demonstrated in different models of pancreatic inflammation, such as L-arginine pancreatitis, pancreatitis induced by taurocholic acid, and by obstruction of the pancreatic duct[110,117,119]. In severe acute pancreatitis, pretreatment with melatonin prevented multiorgan failure and significantly attenuated tissue damage[119]. Melatonin has been shown to increase the nucleic acid content and rate of DNA synthesis in the pancreas and thus to improve pancreatic regeneration in animals subjected to acute pancreatitis[120].

The mechanism of the pancreatic protective action of melatonin is complex. Melatonin works as a direct and indirect antioxidant and effectively reduced the amount of lipid peroxidation products in pancreatic tissue, which is accompanied by the increase of antioxidant enzymes, such as SOD or GPx[98,121]. Melatonin also slows down apoptosis and necrosis in the pancreas and turns down leukocyte infiltration in the pancreatic tissue subjected to acute inflammation. The blood levels of pro-inflammatory interleukin, such as TNFα, IL-1β, IL-6 and IL-22, were reduced, whereas anti-inflammatory IL-10 increased in response to melatonin application[10,105,116,119]. Among the other mechanisms of the pancreatic protection afforded by melatonin is the improvement of pancreatic blood flow, which ameliorates the tissues from the toxic inflammatory products and mediators[108,113]. Melatonin lowers the activity of macrophages and decreases the production of enzyme myeloperoxidase and generation of prostaglandin[104,105]. Melatonin has also been shown to trigger the production of heat shock protein 60 (HSP60) in the pancreatic acinar AR42J cells. Activation of HSP60 by melatonin could protect the intracellular structures against inflammatory damage and could reduce the acinar cell injury[122] (Figure 1).

Melatonin seems to be a part of the natural protective mechanisms against the development of pancreatic inflammation and low melatonin production is associated with an increased risk of severe acute pancreatitis[118,123,124]. Recent reports indicate that the melatonin blood level in humans is closely related to the severity of pancreatitis. It has been proposed that the evaluation

of disease severity could be assessed by measuring the blood levels of melatonin[125].

It is very likely that melatonin might be a part of native protection against the development of acute pancreatitis and a low melatonin blood level could be associated with an increased risk of the severe form of this disease. Perhaps melatonin could be used in the prevention of acute pancreatitis in individuals with an increased risk of pancreatic inflammation.

CONCLUSION

Ghrelin, leptin and melatonin given prior to the induction of experimental acute pancreatitis resulted in the significant attenuation of pancreatitis severity and protected pancreatic tissue against inflammatory damage. The mechanisms of the beneficial influence of the above substances are related to the mobilization of non-specific immune defense, to the inhibition of nuclear NF-κB and the modulation of cytokine production, to the stimulation of heat shock protein, as well as to the activation of the antioxidant system. Experimental and clinical data have shown that blood levels of ghrelin, leptin and melatonin increase in the initial phase of pancreatic inflammation. This observation indicates that these hormones could be a part of the innate resistance system which might remove noxious factors and could suppress or attenuate the inflammatory process in the pancreas.

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2014 ADVANCES IN PANCREATITIS

Balloon dilation itself may not be a major determinant of post-endoscopic retrograde cholangiopancreatography pancreatitis

Sung Ill Jang, Gak Won Yun, Dong Ki Lee

Sung Ill Jang, Department of Internal Medicine, Kangnam Sacred Heart Hospital, Hallym University College of Medicine, Seoul 150-950, South Korea
Gak Won Yun, Dong Ki Lee, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, Seoul 135-720, South Korea
Author contributions: Jang SI reviewed the literature, collected data, wrote and prepared the first draft of this manuscript; Yun GW reviewed the literature, collected data and prepared the tables of this review; and Lee DK designed, reviewed the literature, wrote, revised the paper and performed significant editing.
Correspondence to: Dong Ki Lee, MD, PhD, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, 211 Eonju-Ro, Gangnam-Gu, Seoul 135-720, South Korea. dklee@yuhs.ac
Telephone: +82-2-20193214 Fax: + 82-2-34633882
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Abstract

Endoscopic retrograde cholangiopancreatography (ERCP) is the essential first modality for common bile duct (CBD) stone therapy. The conventional endoscopic treatment for CBD stones is stone removal after endoscopic sphincterotomy (EST). Stone removal after papillary stretching using balloon dilation instead of the conventional method has been widely adopted. There are many reports regarding endoscopic papillary balloon dilation (EPBD) utilizing a small balloon (< 10 mm) instead of EST for the removal of small CBD stones. In contrast, two cases of mortality due to post-ERCP pancreatitis (PEP) were reported after an EPBD clinical trial in the Western world, and the psychological barrier caused by these incidences hinders the use of this technique in Western countries. Endoscopic papillar large balloon dilation (EPLBD), which is used to treat large CBD stones, was not widely adopted when first introduced due to concerns about perforation and severe pancreatitis from the use of a large balloon (12-20 mm). However, as experience with this procedure accumulates, the occurrence of PEP with EPLBD is confirmed to be much lower than with EPBD. This report reviews whether EPBD and EPLBD, two procedures that use balloon dilation but differ in terms of indications and concept, contribute to the occurrence of PEP.

Core tip: Endoscopic papillary balloon dilation (EPBD) and endoscopic papillary large balloon dilation (EPLBD) have been performed for removal of common bile duct stones. Although the rates of post-endoscopic retrograde cholangiopanc- reatography pancreatitis (PEP) after EPBD and EPLBD varied in many studies, the safety and feasibility of balloon dilation have been proven as results have accumulated. However, the exact mechanism of PEP after balloon dilation is unclear. The main determinant of severe PEP may be edema or spasm caused by irritation of the pancreatic orifice while performing difficult selective cannulation and struggling to remove the stone rather than balloon compression of the pancreatic flow.

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INTRODUCTION

Post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP) is a complicating adverse event, for which doctors can do little to treat. Removing common bile duct (CBD) stones is the most frequently performed procedure carried out using ERCP. Conventional endoscopic biliary stone removal through endoscopic sphincterotomy (EST) has been replaced by the balloon dilation method.

PEP, the most common and potentially serious complication of ERCP-related procedures, occurs in 1%-9% of all procedures[1]. Many studies of the risk and predictive factors to prevent PEP have been conducted[1-16]. To discuss the risk factors for PEP, not only procedural and technical factors but also patient characteristics should be considered[1,15]. Patient-related factors for PEP include a history of post-ERCP pancreatitis[1,9,10,12,16,17], female sex[1,10,15], young age[3,4,6,12,15], suspected sphincter of Oddi dysfunction (SOD)[1,2,4,10,12,14], and absence of chronic pancreatitis[1]. Procedural-related factors include difficult cannulation[1,9,15-17], precut sphincterotomy[2,10,15,16], pancreatic deep wire pass[15], pancreatic sphincterotomy[1], pancreatic contrast injections[1-4,10,12,17,18], and biliary balloon sphincter dilation[1]. These reported risk factors vary among studies, and some contradict each other. Hence, the data for risk factors for PEP should be interpreted with caution. Such discrepancies may have arisen from heterogeneous patient populations or from differences in the level of endoscopic expertise, cannulation techniques, and definition of post-ERCP pancreatitis[12].

The PEP mechanism is not well defined, but it is commonly accepted to be multifactorial, involving mechanical, chemical, hydrostatic, enzymatic, microbial, and thermal factors[1,12]. A certain triggering event may prematurely activate proteolytic enzymes intracellularly within acinar cells, which may cause cellular injury and autodigestion of pancreatic tissue[19,20]. Various PEP mechanisms have been suggested. PEP may occur from incidental injection of contrast medium into the pancreatic duct in cases where cannulation of the bile duct is difficult; in such cases, the type of contrast medium injected and the speed and pressure of injection leading to complete acinar filling of the pancreas can have an influence[8,21-23]. Hydrostatic injury caused by pancreatic duct overfilling may be a major trigger factor for pancreatic reactions[16]. Difficult cannulation may inflict trauma to the papilla and pancreatic sphincter, leading to pancreatic drainage disruption and causing PEP[12,24,25]. Pancreatitis after pancreatic sphincterotomy, and precutting have been discussed based on the possibility of incidental temporary obstruction of the pancreatic duct, caused by direct thermal damage to the duct by the cutting wire or by edema induced by thermal tissue injury[8,26]. PEP occurs more frequently in patients with SOD[1,2,10,14,27-29], which may cause a flow disturbance in pancreatic drainage due to pancreatic sphincter hypertension.

Balloon inflation is also a possible cause of PEP. The pancreatic orifice is compressed during ballooning, and pancreatic flow is transiently disrupted. However, it is unknown whether ballooning itself is a major determinant for the development of PEP due to endoscopic papillary balloon dilation (EPBD) and endoscopic papillary large balloon dilation (EPLBD). This report provides a systemic review of how the balloon itself affects PEP in balloon dilation during EPBD and EPLBD.

ENDOSCOPIC PAPILLARY BALLOON DILATION AND ENDOSCOPIC PAPILLARY LARGE BALLOON DILATION

Definition and concepts

The ampullary orifice and distal CBD are dilated temporarily by balloon dilation during EPBD and EPLBD procedures. However, EPBD and EPLBD are not the same procedure in either concept or indications. EPBD is performed in patients with a non- or minimally dilated CBD[30]. In contrast, EPLBD should be performed in patients with dilated CBD and ampulla due to a large long-standing stone. Ballooning of the CBD and ampullary orifice stretches the tissue transiently to form a tubular shape to facilitate stone removal. In this process, successful dilation depends on the elasticity, the degree of dilation of the CBD tissue, and the absence of stricture. Moreover, the ease of selective bile duct cannulation and stone removal through the widened ampullary orifice might be associated with the occurrence of PEP.

EPBD has been performed to dilate the biliary sphincter without prior EST by using a small-diameter dilating balloon ($\leqslant$ 10 mm). EPBD can avoid the short-term complications of bleeding and perforation, preserve the function of the biliary sphincter, and reduce long-term sequelae of EST. Additionally, EPLBD has been used to remove large CBD stones after dilating the biliary sphincter with EST using a large-diameter dilation ($\geqslant$ 12 mm). EPLBD can reduce the use of mechanical lithotripsy (MLT), thereby avoiding complications related to the use of full EST for the removal of large or difficult CBD stones. The majority of EPLBDs involve limited EST (minimal or mid-EST rather than full EST) followed by large balloon inflation. If EST is performed toward the CBD, tearing advances in the same direction[31]. This combined approach does not require full EST and can enlarge the biliary orifice to a greater extent than a standard full EST[30].

Indications

EPBD is a possible alternative to EST in patients with impaired hemostasis[11]. To maximize the effect of EPBD while minimizing complications, a technique with proper indication and in the proper manner is necessary[32]. Recommended indications for balloon dilation include coagulopathy, periampullary diverticulum, Billroth II gastrojejunostomy, and prior EST status[32]. Another study suggested that the ideal patients are those with a limited number of CBD stones ($\leqslant$ 3), CBD stones of a maximum diameter $\leqslant$ 10 mm, and minimally dilated bile

duct[33]. In addition, in cases with difficult cannulation, impractical cannulation should be avoided. The use of EPBD for removing CBD stones > 10 mm may necessitate a laborious and papilla-traumatic procedure, such as MLT, and may increase the risk of pancreatitis[34]. EPBD for a large CBD stone requires multiple sessions and is time-consuming because the biliary orifice is not dilated sufficiently[35]. Therefore, relatively large stones with a non-dilated CBD are not good candidates for EPBD. Further large-scale studies with a longer follow-up are imperative to identify more distinct indications and the optimum method.

Strict indications are needed for EPLBD to avoid serious adverse events. The patients suitable for this method are those who already have a dilated CBD due to a large stone[36]. The tissue of ampulla and distal CBD in these patients are ready to be dilated and further gradual stretching of the tissue will not cause stress or sudden tearing of the ampullary roof. However, patients with the CBD of less than the balloon size or strictures of the distal CBD should be excluded because of the possibility of perforation. The velocity and duration of balloon inflation vary across studies, ranging from a few seconds to minutes. Although guidelines pertaining to the optimal velocity of balloon inflation have yet to be established, the following guidelines for safe EPLBD were proposed based on the current knowledge[36,37]: (1) selection of suitable candidates; i.e., EPLBD should be reserved for patients with a dilated CBD, but avoided in patients with distal CBD strictures; (2) avoidance of full-EST immediately before large balloon dilation to prevent perforation and bleeding; (3) gradual inflation of the dilating balloon to recognize a narrowed distal CBD indicated by lack of disappearance of the balloon waist; (4) discontinuation of inflation when resistance is encountered in the presence of a persistent balloon waist; (5) not inflating the dilation balloon beyond the maximal size of the upstream dilated CBD; and (6) conversion to alternative stone removal or drainage methods when difficulty in removal of a stone is encountered. When a tapered, distal CBD or occult stricture is identified, the operator should pay particular attention to avoiding fatal adverse events caused by large perforations occurring during balloon inflation.

BALLOONING AND PEP

Acute pancreatitis after endoscopic papillary balloon dilation

EPBD, suggested by Staritz et al[38], is an alternative method to EST for removal of CBD stones. EPBD was adopted to reduce the risk of bleeding[39-42] and preserve the function of the sphincter of Oddi[40,43-47]. Some studies have reported that there was no difference in efficacy and safety between EST and EPBD[46,48], whereas others claimed that the incidence of pancreatitis was higher among patients who received EPBD compared with those who received EST[33,49,50]. The results of randomized control studies comparing EPBD and

EST are inconsistent, particularly in terms of the incidence and severity of PEP[46,48-50]. Severe morbidity in the EPBD group compared with the EST group caused early termination of one study[51], and some clinicians assert that EPBD should not be performed based on the pancreatitis-induced mortality that occurred during some studies[50,52]. In particular, another randomized, controlled multicenter trial was also terminated early during the first interim analysis, because two patients died from severe pancreatitis as a complication of EPBD[50]. Thus, the investigation of risk factors for EPBD-related pancreatitis remains controversial.

In studies from Holland[46] and the United Kingdom[48], the incidence rates of pancreatitis with EPBD appeared to be similar to those with EST. In a Japanese study, the pancreatitis rate was slightly higher with EPBD than with EST[49]. In contrast, an American study by Disario et al[50] reported that the post-EPBD pancreatitis rate was higher than that of EST, and mortality was due to pancreatitis. Although EPBD is used less frequently in Western countries due to these complications, it has been continuously adopted in some Japanese groups. There is no clear explanation for this discrepancy, but it is presumed that differences in patient populations and methods of balloon dilation may play a role[42]. In addition, the US study may have included patients with SOD[42]. Thus, pancreatitis-associated EPBD is a very controversial and serious topic[53,54].

The incidence of acute pancreatitis after EPBD ranges from 5%-20%, although most figures fall in the range of 5%-7%[43,46,51]. The frequency and severity of PEP following EPBD are summarized in Table 1. The precise mechanism underlying post-EPBD pancreatitis is not well defined, and appears to be multifactorial. Contrast medium injection into the pancreatic duct[11] and a history of prior pancreatitis[54] are suggested to be risk factors for post-EPBD pancreatitis. Younger age is a risk factor for post-ERCP pancreatitis[3,4,6,12,15], but not for post-EPBD pancreatitis[11,54]. However, in real practice, most cases of severe pancreatitis involve relatively young patients with unatrophied pancreatic tissue.

The mechanism of pancreatitis induced by superfluous injection of contrast medium is regarded as the same as that with EST[11]. Some research suggests that papillary edema or spasm caused by balloon dilation can result in pancreatitis by obstructing pancreatic outflow[11,42]. Balloon compression of the papilla or the pancreatic duct orifice may provoke peripapillary edema and/or spasm of the sphincter of Oddi[51,53]. However, peripapillary trauma by cannulation can more definitely and frequently provoke spasm of the sphincter of Oddi and/or hemorrhagic edematous change[55], and it is a potential risk factor for asymptomatic hyperamylasemia after EPBD[54]. In addition, the biliary orifice may not fully dilate during stone removal with EPBD[56,57]. In this situation, stone removal can be more technically challenging and time-consuming[46,50,58], and subsequent papillary injury or edema during stone extraction can cause pancreatitis[35].

Table 1 Frequency and severity of pancreatitis and complications after endoscopic papillary balloon dilation

Ref.	Study design	Study's location	Comparison groups (n)	Pancreatitis n (%)	Pancreatitis severity (n)			Other complications (n)			Overall AEs-related death (n)
					Mild/ moderate	Severe	Death	Bleeding	Perforation	Cholangitis	
Minami *et al*[85], (1995)	RCT	Japan	EPBD (n = 20)	2 (10)	2	0	0	0	0	0	0
			EST (n = 20)	2 (10)	2	0	0	0	0	0	0
Mathuna *et al*[86], (1995)	R	Ireland	EPBD (n = 100)	5 (4.8)	5	0	0	0	0	0	0
Bergman *et al*[46], (1997)	RCT	The Netherlands	EPBD (n = 101)	7 (6.9)	5	2	0	0	2	0	0
			EST (n = 101)	7 (6.9)	6	1	0	4	1	0	1
Yasuda *et al*[87], (1998)	P	Japan	EPBD (n = 92)	8 (8.7)	8	0	0	0	0	0	0
Ueno *et al*[55], (1999)	R	Japan	EPBD (n = 109)	21 (19.8)	21	0	0	NA	NA	NA	0
Ochi *et al*[88], (1999)	RCT	Japan	EPBD (n = 55)	0	0	0	0	0	0	0	0
			EST (n = 55)	2 (3.6)	2	0	0	0	1	0	0
Arnold *et al*[51], (2001)	RCT	Germany	EPBD (n = 30)	6 (20.0)	4	2	0	0	0	3	0
			EST (n = 30)	3 (10.0)	3	0	0	2	0	0	0
Bergman *et al*[53], (2001)	RCT	The Netherlands	EPBD (n = 93)	7 (7.5)	5	2	0	0	2	0	1
			EST (n = 87)	7 (8.0)	6	1	0	2	0	0	0
Yasuda *et al*[43], (2001)	RCT	Japan	EPBD (n = 35)	2 (5.7)	2	0	0	0	0	0	0
			EST (n = 35)	2 (5.7)	2	0	0	1	0	0	0
Natsui *et al*[89], (2002)	RCT	Japan	EPBD (n = 70)	4 (5.7)	4	0	0	0	0	2	
			EST (n = 70)	3 (4.3)	3	0	0	2	0	3	
Fujita *et al*[49], (2003)	RCT	Japan	EPBD (n = 138)	15 (10.9)	15	0	0	0	0	2	0
			EST (n = 144)	4 (2.7)	4	0	0	2	0	6	0
Vlavianos *et al*[48], (2003)	RCT	United Kindom	EPBD (n = 103)	5 (4.9)	4	1	0	0	0	2	0
			EST (n = 99)	1 (1.0)	1	0	0	0	0	1	1
Sugiyama *et al*[54], (2003)	R	Japan	EPBD (n = 118)	7 (6.0)	7	0	0	0	0	0	0
Lin *et al*[90], (2004)	RCT	Taiwan	EPBD (n = 51)	0	0	0	0	1	0	0	0
			EST (n = 53)	0	0	0	0	14	0	0	0
Disario *et al*[50], (2004)	RCT	United States	EPBD (n = 117)	18 (15.4)	12	6	2	11	0	1	2
			EST (n = 120)	1 (0.8)	1	0	0	32	1	1	0
Tanake *et al*[91], (2004)	RCT	Japan	EPBD (n = 16)	3 (18.8)	1	2	0	0	0	0	0
			EST (n = 16)	3 (18.8)	2	1	0	0	0	2	0
Toda *et al*[92], (2005)	RCT	Japan	EPBD (n = 94)	7 (6.4)	7	0	0	0	0	4	0
			EST (n = 102)	3 (3)	3	0	0	2	2	4	0
Tsujino *et al*[11], (2005)	R	Japan	EPBD (n = 304)	15 (5.0)	15	0	0	0	1	6	0
Nakagawa *et al*[93], (2006)	R	Japan	EPBD (n = 201)	2 (1.0)	0	2	0	0	0	3	0
Tsujino *et al*[42], (2007)	P	Japan	EPBD (n = 1000)	48 (4.8)	47	1	0	2	2	27	2
Ito *et al*[35], (2008)	R	Japan	EPBD (n = 406)	19 (5.7)	19	0	0	0	1	4	0
Liao *et al*[94], (2008)	RCT	Taiwan	EPBD (n = 35)	2 (5.7)	2	0	0	0	0	1	0
			EST (n = 25)	3 (12)	3	0	0	2	0	2	0
Natsui *et al*[95], (2011)	RCT	Japan	EPBD (n = 41)	2 (4.8)	2	0	0	0	0	1	0
			EST (n = 42)	1 (2.3)	1	0	0	0	0	1	0
Kuo *et al*[96], (2012)	R	Taiwan	EPBD (n = 273)	30 (10.1)	22	8	0	1	1	9	1
Seo *et al*[97], (2014)	RCT	South Korea	EPBD (n = 62)	5 (8.1)	5	0	0	0	0	0	0
			EST (n = 70)	5 (7.1)	5	0	0	2	1	0	0

EST: Endoscopic sphincterotomy; AEs: Adverse events; EPBD: Endoscopic papillary balloon dilation; R: Retrospective; P: Prospective; RCT: Randomized controlled trial; NA: Not available.

In particular, stone removal by EPBD becomes more difficult when the stone is large and the use of MLT is more frequent[33,50]. In such cases, the biliary orifice is more likely to be damaged, and the risk of pancreatitis can be greater.

Acute pancreatitis after endoscopic papillary large balloon dilation with EST

EPLBD with limited EST is gradually being recognized as an important modality for the removal of large CBD stones[36,59-67]. Pancreatitis occurs in 2.4% (0%-13.2%) of

patients; almost all cases have been of mild to moderate severity (98.4%)[37,68,69]. The frequency and severity of PEP after EPLBD with EST are summarized in Table 2.

Standard procedural guidelines have not been established; yet, most procedures involve limited EST followed by large balloon inflation. If EST is performed toward the CBD, the direction of tearing advances toward the CBD, and less pressure is applied on the pancreatic duct[31]. It has been suggested that the radial force generated by the dilating balloon is exerted toward the CBD and moves away from the pancreatic duct, which lessens the likelihood of pancreatitis by reducing periampullary injury[31,36,37,62,70]. Moreover, and in contrast to EPBD, EPLBD dilates the ampullary orifice sufficiently to allow for straightforward removal of a large CBD stone, using a Dormia basket or retrieval balloon, and so that it is wide enough to reduce the need for MLT[71]. Additionally, because of the patulous papillary orifice caused by a large stone, endoscopists feel comfortable with selective cannulation of the bile duct in most patients. These are all reasons for decreased occurrence of pancreatitis by reduced ampulla injury, which can cause periampullary trauma or edema[36].

Surprisingly, according to a multicenter retrospective study[37], there was no severe PEP after EPLBD among 946 patients. This provides strong evidence that ballooning itself is not a major determinant of PEP in EPLBD. Moreover, according to a systemic review of EPLBD, even though the inclusion criteria and procedure type were heterogeneous[69], PEP following EPLBD was not problematic. The duration of ballooning in EPLBD is usually 30-60 s in real practice. However, the timing of balloon inflation is not related to PEP[30,69], so prolonged balloon inflation does not increase PEP in EPLBD.

Acute pancreatitis after EPLBD without EST

EPLBD without EST is preferred in patients with bleeding tendencies, altered anatomy, and, in some cases, periampullary diverticulum[34]. If EST is not performed prior to balloon application, theoretically, the PEP rate may increase because pancreatic outflow could be more completely obstructed by the balloon. In addition, the balloon could press the pancreatic orifice from a more acute angle than when the papillary roof incision is made, because the biliary and pancreatic orifices are not separated. However, according to a retrospective analysis[30] and systematic review[69], the PEP rate is not high and does not differ between EPLBD with and without EST. Moreover, the incidence of PEP did not change with ballooning time[68]. Therefore, ballooning during EPLBD is not a major factor for PEP, regardless of whether EST is performed.

Some recent studies have reported that EPLBD without EST is safe and effective in patients with large CBD stones[34,72,73]. Pancreatitis and bleeding occurred at a rate of 0.8%-6.5%, and all cases were of mild to moderate severity. The frequency and severity of PEP after EPLBD without EST are summarized in Table 3. This is sup-ported by a large-scale study reporting less frequent pancreatitis resulting from a larger balloon[37]. In other words, the extent of biliary orifice dilation is relevant to the incidence of pancreatitis, rather than the size of the balloon, EST performance, or balloon dilation time[65,68,73].

DISCUSSION

Although EPBD involves a high incidence of pancreatitis, the reports are inconsistent, and it remains controversial. In studies with high rates of pancreatitis, a discrepancy in patient selection should have been made before suggesting balloon dilation as the primary risk factor for pancreatitis. The reason for the high incidence of pancreatitis in EPBD is that enables removal of only small-to-medium sized stones, and patients with such a stone size tend to possess the known risk factors for pancreatitis: young age, non-dilated CBD, normal pancreas parenchyma, obesity, and SOD dysfunction. In other words, careful patient selection can lessen the risk of post-ERCP pancreatitis. EPBD for larger CBD stones, rather than a non-dilated CBD, requires multiple sessions and is more time consuming for stone removal than EST, because EPBD cannot dilate the biliary orifice sufficiently[35]. Baron *et al*[33] recommended extreme caution when performing EBPD in patients with severe acute cholangitis, a history of previous or ongoing acute pancreatitis, age ≤ 50 years, and difficult biliary cannulation. To prevent post-ERCP pancreatitis, pancreatic duct stent insertion is also recommended when EPBD is performed in young patients[33].

The frequency or severity of PEP after EPBD did not vary with ballooning time. This implies that balloon compression of the pancreatic orifice for < 1 or 2 min without stimulated pancreatic secretion does not cause significant pancreatitis. In one randomized prospective study[74], 5-min EPBD reduced the risk of pancreatitis compared with conventional 1-min EPBD. Rather than ballooning itself, we believe that pancreatic edema or spasm caused by papillary irritation due to difficult selective cannulation and forcible stone extraction might be the major determinant of PEP after EPBD.

During the early period of EPLBD, PEP is the main concern, because the pancreatic orifice is compressed with a balloon larger than that used in EPBD. However, accumulated data inform clinicians that the larger balloon does not result in PEP, although in practice, one case of severe pancreatitis with mortality has been reported[75]. Although the major etiological factors of pancreatitis and its mechanism remain unclear, the mechanism of pancreatitis may differ between EPLBD and EPBD. EPLBD and EPBD are different procedures clinically. The major difference is that EPLBD cannot be applied to a non-dilated bile duct, which can be a risk factor for PEP[54]. If the orifice is sufficiently dilated by EPLBD, papillary edema or spasm is less likely to occur due to use of a basket or retrieval balloon catheter, unlike EPBD, and the incidence of pancreatitis may decline due to the less frequent

Table 2 Frequency and severity of pancreatitis and complications after endoscopic papillary large balloon dilation with endoscopic sphincterotomy

Ref.	Study design	Study's location	Comparison groups (*n*)	Balloon diameter (mm)	Pancreatitis *n* (%)	Pancreatitis severity (*n*)			Other complications (*n*)			Overall AEs-related death (*n*)
						Mild/ moderate	Severe	death	Bleeding	Perforation	Cholangitis	
Ersoz *et al*[31], (2003)	R	Turkey	EPLBD (*n* = 58)	12-20	2 (3.4)	2	0	0	5	0	2	0
Maydeo *et al*[60], (2007)	P	India	EPLBD (*n* = 60)	12-20	0 (0)	0	0	0	5	0	0	0
Minami *et al*[62], (2007)	R	Japan	EPLBD (*n* = 88)	20	1 (1.1)	1	0	0	1	0	1	0
Heo *et al*[61], (2007)	RCT	South Korea	EPLBD (*n* = 100)	12-20	4 (4.0)	4	0	0	0	0	0	0
			EST (*n* = 100)	-	4 (4.0)	4	0	0	2	0	0	0
Lee *et al*[98], (2007)	R	South Korea	EPLBD (*n* = 55)	15-20	0 (0)	0	0	0	2	0	0	0
Kim *et al*[99], (2007)	R	South Korea	EPLBD (*n* = 35)	12-20	0 (0)	0	0	0	0	1	0	0
Lee *et al*[100], (2007)	R	South Korea	EPLBD (*n* = 41)	13-20	2 (4.8)	0	0	0	1	0	0	0
Misra *et al*[101], (2008)	R	India	EPLBD (*n* = 50)	15-20	4 (8.0)	4	0	0	3	0	0	0
Attasaranya *et al*[63], (2008)	R	United States	EPLBD (*n* = 103)	12-18	0 (0)	0	0	0	2	1	0	0
Espinel *et al*[102], (2008)	P	Spain	EPLBD (*n* = 93)	12-20	1 (1.1)	1	0	0	1	0	0	0
Itoi *et al*[103], (2009)	R	Japan	EPLBD (*n* = 53)	15-20	1 (1.9)	1	0	0	0	0	1	0
			EST (*n* = 48)	-	2 (4.1)	2	0	0	0	0	1	0
Kim *et al*[104], (2009)	RCT	South Korea	EPLBD (*n* = 27)	15-18	0 (0)	0	0	0	4	0	0	0
			EST (*n* = 28)	-	0 (0)	0	0	0	2	0	0	0
Itoi *et al*[105], (2010)	R	Japan	EPLBD (*n* = 18)	15-18	0 (0)	0	0	0	0	0	0	0
Kurita *et al*[106], (2010)	R	Japan	EPLBD (*n* = 24)	15-20	0 (0)	0	0	0	0	0	0	0
Ghazanfar *et al*[107], (2010)	P	Pakistan	EPLBD (*n* = 84)	15-18	3 (3.6)	3	0	0	3	0	0	1
Kim *et al*[108], (2010)	R	South Korea	EPLBD (*n* = 70)	12-18	1 (2.3)	1	0	0	0	0	0	0
Youn *et al*[65], (2011)	R	South Korea	EPLBD (*n* = 101)	15-20	2 (2.0)	2	0	0	2	1	0	0
Kim *et al*[109], (2011)	R	South Korea	EPLBD (*n* = 72)	12-20	5 (6.9)	5	0	0	0	0	1	0
			EST (*n* = 77)	-	9 (11.7)	9	0	0	0	1	0	0
Stefanidis *et al*[75], (2011)	RCT	Greece	EPLBD (*n* = 45)	15-20	1 (2.2)	1	0	0	1	0	0	0
			EST (*n* = 45)	-	1 (2.2)	1	0	0	1	1	6	0
Rebelo *et al*[67], (2012)	R	Portugal	EPLBD (*n* = 30)	12-18	1 (3.3)	1	0	0	0	0	0	0
Sakai *et al*[110], (2013)	R	Japan	EPLBD (*n* = 59)	12-20	0 (0)	0	0	0	1	1	0	0
Park *et al*[37], (2013)	R	South Korea, Japan	EPLBD (*n* = 946)	12-20	24 (25.3)	24	0	0	56	9	6	4
Poincloux *et al*[64], (2013)	R	France	EPLBD (*n* = 64)	15-20	2 (3.1)	2	0	0	5	0	0	0
Hwang *et al*[73], (2013)	R	South Korea	EPLBD (*n* = 69)	12-20	3 (4.3)	3	0	0	0	1	0	0
Paspatis *et al*[68], (2013)	RCT	Greece	60 s dilation[1] (*n* = 60)	15-20	2 (1.6)	2	0	0	4	1	3	0
			30 s dilation[1] (*n* = 64)	15-20	2 (1.6)	1	1	1	2	1	2	1
Rosa *et al*[66], (2013)	R	Portugal	EPLBD (*n* = 68)	12-18	9 (13.2)	9	0	0	0	0	1	0
			EST (*n* = 45)	-	2 (4.7)	2	0	0	0	0	1	0

Yang et al[111], (2013)	R	China	EPLBD (n = 171)	12-18	2 (1.2)	2	0	0	4	1	1	0
Yoon et al[112], (2013)	P	South Korea	EPLBD (n = 52)	12-20	0 (0)	0	0	0	0	0	0	0
Harada et al[113], (2013)	R	Japan	EPLBD (n = 30)	15-20	0 (0)	0	0	0	0	0	0	0

[1]Sixty and thirty seconds mean the duration of balloon dilation (s) in endoscopic papillary large balloon dilation (EPLBD). AEs: Adverse events; EST: Endoscopic sphincterotomy; R: Retrospective; P: Prospective; RCT: Randomized controlled trial.

Table 3 Frequency and severity of pancreatitis and complications after endoscopic papillary large balloon dilation without endoscopic sphincterotomy

Ref.	Years	Study design	Patients (n)	Pancreatitis n (%)	Pancreatitis severity (n)				Other complications (n)			Overall AEs-related death (n)
					Mild	Moderate	Severe	Death	Bleeding	Perforation	Cholangitis	
Jeong et al[34]	2009	R	38	1 (2.6)	1	0	0	0	0	0	0	0
Chan et al[72]	2011	R	247	2 (0.8)	2	0	0	0	0	0	1	0
Hwang et al[73]	2013	R	62	4 (6.4)	4	0	0	0	0	0	0	0

AEs: Adverse events; R: Retrospective.

use of MLT[34,72]. There is no significant difference in the frequency of the requirement for MLT between EPLBD and the conventional method. However, adequate fragmentation of the CBD stone by MLT after EPLBD can reduce the frequency of the requirement for MLT in EPLBD compared with the conventional method[69]. This could be one reason for the lower incidence of PEP in EPLBD. Patients who receive EPLBD are relatively older individuals in whom pancreatic exocrine function has declined, and pancreatitis is less likely[72]. Further, easy selective cannulation into the bile duct can reduce the incidence of pancreatitis. Additionally, contrary to our concern, EPLBD without a preceding papillary incision did not cause severe pancreatitis[34,72,73]. Therefore, ballooning itself may not be the culprit. And the cause of fatal pancreatitis during EPBD should be reconsidered.

The incidence of pancreatitis, using percutaneous papillary balloon dilation (PTPBD) for CBD stone removal, is extremely low (0%-1.4%)[76-82]. A retrospective study reported that pancreatitis occurred only in the EPBD group; in another study, comparing PTPBD with EPBD, the only significant predictor was the use of MLT[76]. The size of the balloon used in PTPBD varies (4-23 mm) among studies and can also vary within the same study, due to the presence of differently sized CBD stones (5-20 mm)[77-82]. In one study[76], patients with a CBD stone < 12 mm in diameter were enrolled homogenously, and the balloon dilation diameter was 8-10 mm, which was compatible with EPBD. The largest balloons used for papillary dilation were of diameters 22 mm[80] and 23 mm[82] in other studies; this balloon inflation size is compatible with EPLBD. Although the balloon dilation diameter was different in each study, no severe pancreatitis occurred. These studies confirmed that ballooning does not increase the incidence of PEP. Moreover, the rates of post-procedural pancreatitis and hyperamylasemia were significantly higher following retrograde dilation using EPBD, compared with antegrade dilation using PTPBD, during the removal of bile duct stones[76].

The reason for the lower rate of PEP with antegrade application of balloon inflation compared with a retrograde fashion is the lack of difficulty in selective cannulation and lower chance of difficult procedure for forcible removal unless the stone descends. Compared with EPBD, PTPBD inflicts less mechanical trauma to papilla during stone removal, and it is nearly equivalent to the effect of ballooning[83,84]. In addition, MLT application does not involve lithotripsy moving back and forth through the ampullary orifice, in which there is no chance of pancreatic orifice damage. Such a result demonstrates that ballooning may not be a risk factor for pancreatitis. Moreover, the rates of post-procedural pancreatitis and hyperamylasemia were significantly higher after retrograde dilation with EPBD than after antegrade dilation with PTPBD for the removal of bile duct stones. This reveals that pancreatitis can be induced by other factors, such as repeated cannulation or pancreatic duct injection, during retrograde dilation with EPBD.

CONCLUSION

Although the mechanism of PEP is unclear, the occurrence of pancreatitis is more associated with the catheter, basket, or MLT causing ampullary injury. Instead of balloon compression of the pancreatic flow, the main determinants of severe pancreatitis during endoscopic stone removal with balloon dilation may involve edema or spasm caused by irritation of the pancreatic orifice while performing difficult selective cannulation and struggling to remove the stone. Therefore, ballooning itself may not be the culprit for PEP in either EPBD or EPLBD.

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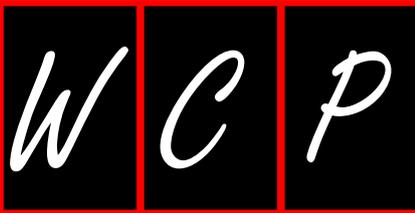
2014 ADVANCES IN PANCREATITIS

Is necrosectomy obsolete for infected necrotizing pancreatitis? Is a paradigm shift needed?

Yu-Chung Chang

Yu-Chung Chang, Department of Surgery, Chung Shan Medical University Hospital, College of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan
Author contributions: Chang YC researched and wrote the manuscript for the Highlight Topic: "Necrotizing Pancreatitis."
Correspondence to: Yu-Chung Chang, MD, PhD, Department of Surgery, Chung Shan Medical University Hospital, College of Medicine, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, South Dist., Taichung 40201,
Taiwan. changmdphd@yahoo.com
Telephone: +886-4-24739595 Fax: +886-4-24756437
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Abstract

In 1886, Senn stated that removing necrotic pancreatic and peripancreatic tissue would benefit patients with severe acute pancreatitis. Since then, necrosectomy has been a mainstay of surgical procedures for infected necrotizing pancreatitis (NP). No published report has successfully questioned the role of necrosectomy. Recently, however, increasing evidence shows good outcomes when treating walled-off necrotizing pancreatitis without a necrosectomy. The literature concerning NP published primarily after 2000 was reviewed; it demonstrates the feasibility of a paradigm shift. The majority (75%) of minimally invasive necrosectomies show higher completion rates: between 80% and 100%. Transluminal endoscopic necrosectomy has shown remarkable results when combined with percutaneous drainage or a metallic stent. Related morbidities range from 40% to 92%. Single-digit mortality rates have been achieved with transluminal endoscopic necrosectomy, but not with video-assisted retroperitoneal necrosectomy series. Drainage procedures without necrosectomy have evolved from percutaneous drainage to transluminal endoscopic drainage with or without percutaneous endoscopic gastrostomy access for laparoscopic instruments. Most series have reached higher success rates of 79%-93%, and even 100%, using transcystic multiple drainage methods. It is becoming evident that transluminal endoscopic drainage treatment of walled-off NP without a necrosectomy is feasible. With further refinement of the drainage procedures, a paradigm shift from necrosectomy to drainage is inevitable.

Core tip: A shift from early, prompt surgical necrosectomy to delay until liquefaction has become the global consensus for treatment of infected necrotizing pancreatitis, which allows drainage procedures and minimally invasive techniques to play a more important role before definitive surgery. Success rates of 80% and single-digit mortality rates are reported with transluminal endoscopic drainage and irrigation with a percutaneous gastrostomy access route. Zero mortality using transluminal endoscopic drainage without a necrosectomy can be achieved. A paradigm shift from necrosectomy to drainage for the treatment of walled-off necrotizing pancreatitis should be considered.

INTRODUCTION

Bradley[1] described the original 19th-century dispute

between Senn and Fitz concerning the value of necrosectomy for necrotizing pancreatitis (NP). In 1886, Senn[2] claimed that removing necrotic pancreatic and peripancreatic tissue would be beneficial for patients with severe acute pancreatitis (AP). Fitz[3], however, was convinced that the prognosis of an individual episode of AP was determined only by the pathologic findings and not based on whether surgical debridement had been performed. But even Fitz[4], who initially considered the operation useless and hazardous, later suggested that the "the sooner the operation was carried out, the better for patients with AP". Until about 1925, Senn's views held sway, and debridement was common practice before AP surgery. Moynihan[5] expressed the prevailing surgical opinion of the time: "Recovery from the disease, apart from operation, is so rare that no case should be left (surgically) untreated. However, few survived surgical intervention. After the development of an amylase assay, the therapeutic pendulum swung away from surgery toward nonsurgical management and surgical intervention was contraindicated. Even though this conservative approach spared the majority of patients with mild or moderate AP surgical intervention, many patients with severe AP still died. In an attempt to confront persistent high mortality rates from nonsurgical management of severe AP, the concepts of surgical approaches were advocated and surgical mortality often exceeded 50%".

Since then, the rationale of AP surgery evolved from exploratory laparotomy to total pancreatectomy in severe AP in the late 1960s and 1970s, to early immediate surgical intervention in the 1980s when the pancreas was proved to be infected, to the notion, in 1993, of 100% mortality if AP was treated non-operatively[6], to the present concept, expressed in 2007, that patients with severe NP complicated with infection benefit from delayed necrosectomy and drainage[7].

Surgical necrosectomy was the mainstay of NP treatment a decade ago, especially when non-surgical approaches failed. However, from serendipitous antibiotic treatment for infected NP[8], non-surgical therapy for sterile NP[1], and no debridement with minimally invasive left-flank drainage[9], to the dual drainage of endoluminal and percutaneous approaches[10], more evidence has been reported for successful treatment of infected NP (INP) without a necrosectomy. This resurrects the same question 125 years later by the author and by Smadja and Bismuth: Is necrosectomy a "useless and hazardous approach" for AP[11]? The same dispute occurred between Bradley[12] and Warshaw[13] over sterile NP but not INP. Bradley concluded: "However, surgical debridement and drainage remains the preferred approach for infected pancreatic necrosis despite occasional anecdotal reports of successful management by transcutaneous or endoscopic means".

Currently, the management of NP has undergone a paradigm shift toward minimally invasive techniques for necrosectomy, obviating the need for open necrosectomy in most cases[14]. There is increasing evidence that minimally invasive approaches, including a step-up approach that incorporates percutaneous catheter or endoscopic transluminal drainage followed by video-assisted retroperitoneal or endoscopic debridement[15], are associated with improved outcomes over traditional open necrosectomy for patients with INP. A recent international multidisciplinary consensus conference emphasized the superiority of minimally invasive approaches over standard surgical approaches[16]. A minimally invasive necrosectomy is still used, according to most reports. Recently, increasing evidence on the efficacy of endoscopic technique includes reports of successes without a necrosectomy when treating NP, which raises the same old question of whether necrosectomy is obsolete.

Purpose: Author's questions
Is necrosectomy mandatory for treating NP? Can we avoid it? If necrosectomy can be avoided, then a paradigm shift may allow us to move toward minimally invasive drainage procedures. Our aim is the same as Traverso and Kozarek[17], who stated that the word "necrosis" induces a "knee-jerk" response to perform necrosectomy. They claimed that, given time, the necrosis would dissolve ("necrolyse") or become infected. Even though INP indicates surgical debridement in most medical centers worldwide, they first perform percutaneous drainage, which, they say, has "drastically lowered the need for pancreatic necrosectomy to less than 10%" and the mortality rate to "single digits".

Data collection
The outcomes of NP, primarily INP, treated using conservative treatment, open necrosectomy, interventional drainage, and minimally invasive methods that were reported after 2000 were reviewed. Morbidity, mortality, reoperation rate, pancreatic fistula rate (for surgery), endoscopic sessions, completion rate for endoscopic methods, and the success rate for drainage methods were compared to see whether there has been a paradigm shift from surgery to minimal invasive-especially drainage-alternatives.

Outcomes
Even for INP, completely conservative treatment (Table 1) with antibiotics without mortality was possible in three reports[8,18,19]. Surgery could be avoided in 67.0%-87.5% of cases. For sterile NP, the mortality of conservative treatment remained between 0% and 15.3% (Table 1), which is the same as reported before 2000.

Despite some studies' reports of single-digit mortality using surgical necrosectomy[20-23], high mortality (20.0%-63.9%) is reported in the majority of series (Table 2). Except in a few centers, surgical outcome has not changed much, and the surgical risk is high. A nationwide study in the United States of 1783 patients from 1998 to 2010 indicated that the incidence of pancreatic debridement significantly decreased from 0.44% to 0.25% and that in-hospital mortality (overall 22.0%) significantly de-

Ref.	Year	Cases (n)	Type (Intend)	Morbidity (%)	Reoperation rate (%)	Mortality (%)	Remark
Sterile							
Bradley[1]	1991	40	Nonsurgical			10.0	
Uomo et al[61]	1996	146	Nonsurgical			9.5	1984-1993
Baril et al[20]	2000	26	Antibiotics		0	7.0	1993-1997
Büchler et al[62]	2000	56	Conservative			1.8	Two false negatives excluded
Zerem et al[41]	2009	20	Conservative		15.0	0.0	Randomized controlled study
Garg et al[54]	2010	137	Conservative			15.3	1997-2008
van Santvoort et al[18]	2011	386	Conservative			7.3	21 Dutch hospitals
Babu et al[46]	2013	14	Conservative			7.0	Step-up; one INP
Infected							
Dubner et al[8]	1996	3	Antibiotics	0	0	0.0	Unstable or refused surgery
Baril et al[20]	2000	6	Antibiotics		0	33.0	1993-1997
Runzi et al[63]	2005	16	Antibiotics	62		12.5	
Lee et al[19]	2007[1]	8	Antibiotics	0	0	0.0	
Garg et al[54]	2010[1]	71	Conservative or PCD			25.4	1997-2008
van Santvoort et al[18]	2011	11	Antibiotics	0	0	0.0	21 Dutch hospitals
Sterile + infected							
Büchler et al[62]	2000	58	Conservative			5.0	Two false negatives
Garg et al[54]	2010[1]	208	Medical			18.8	1997-2008

[1]Non-randomized controlled study. INP: Infected necrotizing pancreatitis; PCD: Percutaneous drainage.

creased from 29.0% to 15%[24].

In the majority (75%) of the included series on minimally invasive necrosectomy report higher completion rates between 80%-100%[10,25-38] (Table 3). Minimally invasive necrosectomy, mainly transluminal endoscopic necrosectomy (TEN) with drainage, has shown remarkable results combined with percutaneous drainage (PCD)[10,26,29,34] or using a metallic stent[28,32]. Related morbidities ranged from 40% to 92%[11,26,30,33,34,36,39,40]. Single-digit mortality rates have been achieved in the majority of the TEN groups, but not in the video-assisted retroperitoneal drainage group. The percutaneous endoscopic gastrostomy access route was used in three series[25,32,34] (Table 3).

The success rate of PCD varies (Table 4). Some series[41-43] report that it remains unchanged at 35%-49%, but most[17,19,20,41] have reached a higher success rate of 76%-93%. The transluminal endoscopic drainage (TED) rates are about 80%[19,44], and even 100%[45] when using single transluminal gateway transcystic multiple drainage methods. Single-digit mortality was reported in most series[19,20,41,44-47], and zero mortality is a reality[19,20,41,45,47].

DISCUSSION

The pathophysiology of AP is usually divided into three phases. In phase one, trypsin is prematurely activated pancreatic acinar cells, which synthesize, store, and secrete digestive enzymes. Once trypsin is activated, it activates a variety of harmful pancreatic digestive enzymes. In phase two, intrapancreatic inflammation occurs through a variety of mechanisms and pathways. In phase three, extrapancreatic inflammation, including acute respiratory distress syndrome occurs, which is often fatal. In about 80% of patients, AP is mild; however, in 10%-20%, the pathways that contribute to increased intrapancreatic and extrapancreatic inflammation lead to

systemic inflammatory response syndrome, a complex response to infection, trauma, burns, pancreatitis, and a variety of other injuries. In some instances, systemic inflammatory response syndrome predisposes a patient to multi-organ dysfunction, pancreatic necrosis, or both[7]. The following precepts have been proposed over the past 130 years: (1) 1886, removing necrotic pancreatic and peripancreatic tissue is beneficial for patients with severe AP[2]; (2) 1889, the sooner surgery is done, the better for patients with AP[3]; (3) 1925, recovering from AP without surgery is rare; thus, no patient with AP should be surgically untreated[5]; (4) 1993, the mortality in non-operatively treated patients approaches 100%[6]; (5) when the pancreas is infected, surgery is mandatory; (6) when the pancreas is infected, early necrosectomy and drainage are recommended; (7) delay until demarcation (used for the era of open necrosectomy to delay the operation timing and to spare the viable pancreatic tissue from being sacrificed during debridement); and (8) 1996, surgical debridement is rarely necessary in sterile pancreatic necrosis[1].

There is no reason to use immediate surgery for patients with mild AP. Infected pancreatic necrosis, however, is an indication for surgical intervention. Approximately 20% of patients develop NP, which has a mortality rate of 15%. The major cause of death, in addition to early organ failure, is extrapancreatic infection or infectious pancreatic necrosis, which leads to sepsis and multi-organ failure. Secondary infection of pancreatic necrosis develops in approximately 30% of patients with necrosis, which increases the mortality rate to approximately 39%. Infected necrosis is virtually always an indication for intervention[48]. Surgery within the first 14 d of the onset of INP should be avoided because early surgery is associated with increased mortality[49]. The conventional management of INP is open surgical debridement. Other surgical approaches have been used,

Table 2 Results of surgical necrosectomy for necrotizing pancreatitis published mainly after 2000

Ref.	Year	Cases (*n*)	Type (Intend)	PF (%)	Morbidity (%)	Reoperation rate (%) or *n*/patient	Mortality (%)	Remark
Sterile								
Baril *et al*[20]	2000	1	Open	0.0	0.0	0.0	0.0	1993-1997
Büchler *et al*[62]	2000	1	Closed+irrigation	?		?	100.0	
Rau *et al*[64]	2005	142	Closed+irrigation	23.0	61.0	43.0	23.0	1992-2001
Howard *et al*[21]	2007	23	Planned re-lap		78.0	30.0	9.0	Symptomatic
Garg *et al*[54]	2010	9	Closed+irrigation	> 30.0			55.5	1997-2008
Infected								
Baril *et al*[20]	2000	11	Open	NA	91.0	45.5	9.0	1993-1997
Büchler *et al*[62]	2000	27	Closed+irrigation	29.0		22.0	18.5	two un-OP excluded
Rau *et al*[64]	2005	140	Closed+irrigation	30.0	78.0	27.0	27.0	1992-2001
Howard *et al*[21]	2007	66	Surgery		86.0	33.0	15.0	
Garg *et al*[54]	2010	36	Closed+irrigation		63.0		63.9	1997-2008
van Santvoort *et al*[15]	2010	45	Closed+irrigation	38.0	69.0	31.0	16.0	RCT; one operation, 42%; 33% need PCD
		24	VARD		65.0		17.0	
van Santvoort *et al*[18]	2011	78	VARD/TEN/OP		64.0		18.0	21 Dutch hospitals
Babu *et al*[46]	2013	27	Closed+irrigation	22.2	51.9	22.2	40.7	Step-up
Sterile + infected								
Smadja and Bismuth[11]	1986	12	Surgery, early				100.0	
		15	Surgery, late				27.0	
		11	Surgery, elective				0.0	
Connor *et al*[39]	2005	47	Closed+irrigation		95.0		39.0	81% infected
Olakowski *et al*[65]	2006	144	Open packing		43.0	3-8/patient	21.0	83% infected
Nieuwenhuijs *et al*[66]	2003	38	Open packing	21.0	89.0	3-70/patient	47.0	
		21	Closed+irrigation	14.0	44.0	0-3/patient	33.0	
Reddy *et al*[40]	2006	118	Closed+irrigation	36.0	58.0	22.9	38.1	65.3% infected
Howard *et al*[21]	2007	102	Planned re-lap					
	(1993-2001)	59			49.0	89.0	67.0	18.0	76% infected
	(2002-2005)	43			60.0	72.0	68.0	4.0	72% infected
Rodriguez *et al*[67]	2008	167	Closed packing	50.0		11.0	11.0	72% infected
Garg *et al*[54]	2010	45	Closed+irrigation				48.9	1997-2008
Babu *et al*[68]	2010	28	PCD + surgery				22.0	2000-2008
Doctor *et al*[22]	2011	61	Open + laproscopy (re-OP 8%)	50.8			9.8	1998-2009; 83.6% INP
Bausch *et al*[50]	2012	30	Closed+irrigation	16.7	90.0	73.3	63.3	83.3% infected
Madenci *et al*[23]	2014	68	Closed packing	74.2	> 74.2	14.7	8.8	2006-2009; 63% infected
Wormer *et al*[24]	2014	1783	Surgical debridement				22.0	1998-2010; nationwide

INP: Infected necrotizing pancreatitis; OP: Operation; PCD: Percutaneous drainage; PF: Pancreatic fistula; RCT: Randomized control study; Re-lap: Re-laparotomy; TEN: Transluminal endoscopic necrosectomy; un-OP: Not operated on; VARD: Video assisted retroperitoneal debridement.

including single-stage and multistage methods with a variety of drainage and closure techniques. Necrosectomy is a relatively standardized technique used with a variety of methods to control drainage, for example, marsupialization of the lesser sac, wide closed-suction drainage, continuous lavage of the septic cavity, and a planned repeat necrosectomy with a delayed primary closure. Less invasive methods have also been reported, namely, using laparoscopic techniques and equipment along the track of existing percutaneous drains[49].

The term and concept of "delay until liquefaction" was developed by the author[9] for minimally invasive drainage from the left flank without debridement. Typically, at least three weeks is needed for liquefaction of the retroperitoneal and peripancreatic tissue to reach the left flank. This permits a sump drain to be inserted from the left flank to the pancreatic head area without opening the abdomen. This strategy is currently commonly used for the timing of delayed management with open or minimally invasive approaches for drainage and necrosectomy[7]. Walling-off the liquefied necrotic tissue that has formed a secure attachment to the gastric or duodenal wall enables endoscopic drainage with or without a necrosectomy from the stomach, duodenum, or left retroperitoneum. A prolonged delay may cause unnecessary adverse events.

Consensus ON NP

Several important points were established at a one-day meeting held in conjunction with the annual meeting of the American Pancreatic Association in 2010[16]: (1) sterile acute necrotic collections almost never require intervention early in the course of disease, and in the later phase (*i.e.*, after several weeks), only if there are disabling symptoms, such as abdominal pain, significant mechanical obstruction (*e.g.*, a gastric or biliary outlet), or both; (2) infected acute necrotic collections may occasionally require early intervention, but because early open surgery is

Table 3 Results of minimal invasive necrosectomy for walled-off necrotizing pancreatitis published after 2000

Ref.	Year	Cases (n)	Type (Intend)	Sessions[1]	Completion rate (%)	Morbidity (%)	Reoperation rate (%)	Mortality (%)	Remark
Infected									
Raczynski et al[25]	2006	2	TEND + irrigation	3	100.0	0.0	0.0	0.0	2 PEG (1st report?)
Escourrou et al[26]	2008	13	TEND + irrigation	1-3	100.0	46.0	0.0	0.0	+ PCD × 2
Bala et al[27]	2009	8	Lt RPD + N+ irrigation	3-17	87.5	25.0		12.5	Stepped
Antillon et al[28]	2009	1	TEN + stent		100.0	0.0	0.0	0.0	Transgastrostomy; Foley irrigation
Will et al[29]	2012	18	TEN ± PCD	3-8	100.0	16.6	0.0	0.0	One unrelated death
Bakker et al[30]	2012	10	TEND	2-6	100.0	20.0	0.0	10.0	RCT; 10% PF
		10	VARD/Lap	1-2	40.0	80.0	60.0	40.0	70% PF
Castellanos et al[31]	2013	32	VARD	1.0	100.0	9.3	0.0	15.6	
Sarkaria et al[32]	2014	17	TEN + Stent ± PEG-J + irrigation	5.3	88.0	5.9	11.8	0.0	8 PEG-J
Sterile + infected									
Connor et al[39]	2005	47 (NS)	Lt. RPD + N	1-9		92.0		19.0	81% INP
Voermans et al[33]	2007	25	TEND + irrigation	NA	92.0	40.0	4.0	0.0	76% NP
Papachristou et al[34]	2007	53	TEND ± PCD ± PEG + irrigation	3	81.0	49.0	22.6	6.0	49% INP
Seifert et al[35]	2009	93 (NS)	TEND	6	80.0	26.0	11.8	7.5	1999-2005
Raraty et al[36]	2010	137 (NS)	VARD			75.0		19.0	64% INP
van Santvoort et al[15]	2010	43 (NS)	Lt. RPD + N (Step-up)	1-7	35.0	40.0	60.0	19.0	RCT
Gardner et al[37]	2011	104	TEND	1-14	91.0	14.0	2.0	2.0	39% INP
Bausch et al[50]	2012	30	PCD + N		57.0	43.0	21.0	21.0	93% INP
		18	TEND		50.0	44.0	28.0	6.0	72% INP
Ross et al[10]	2014	117	TEND + PCD	NA	100.0	4.2	0.0	3.4	Dual modality
van Brunschot et al[38]	2014	455 (NS)	TEN ± PCD	4 (1-23)	81.0	36.0	10.0	6.0	Systematic review; 57% INP

[1]Values are mean or range. INP: Infected necrotizing pancreatitis; Lap: Laparotomy; N: Necrosectomy; NA: Not available; NS: Walled-off necrosis was not specified; PF: Pancreatic fistula; PCD: Percutaneous drainage; PEG-J: Percutaneous endoscopic gastrostomy-jejunal arm; RCT: Randomized control study; RPD: Retroperitonium percutaneous endoscopic gastrostomy; TEN: Transluminal endoscopic necrosectomy; TEND: Transluminal endoscopic necrosectomy with drainage; VARD: Video assisted retroperitoneal debridement; WON: Walled-off necrosis.

associated with high morbidity and mortality, it should be avoided whenever possible. Instead, radiologic or endoscopic drainage should be used before surgery to treat the infection and to postpone or obviate the need for surgical debridement; (3) intervention by any method is optimal when infected necrosis is walled-off and demarcated with at least partial liquefaction and discrete encapsulation. This typically requires a delay of four to six weeks; (4) asymptomatic walled-off necrosis (WON) does not require intervention regardless of the size and extension of the collection; it may eventually resolve spontaneously, even in rare cases of infected necrosis; and (5) symptomatic WON generally requires intervention late in the course (i.e., after four weeks) if there is intractable pain, visceral obstruction (e.g., the stomach or bile duct), or infection.

2013 updated guideline
The optimal management of NP continues to evolve. A 2013 guideline published by the American College of Gastroenterology regarding debridement of necrosis and minimally invasive management of pancreatic necrosis states that the mortality of infected necrosis was falsely believed to be almost 100% in patients with INP not

given immediate surgery[48]. There is ample evidence that antibiotic treatment alone can resolve the infection and, in some patients, preclude surgery. Therefore, the notion that immediate surgery is necessary for patients with INP is no longer valid. Asymptomatic pancreatic and extrapancreatic necrosis do not require intervention regardless of size, location, and extension, because they are likely to spontaneously resolve, even if infected[16]. Unstable patients with infected necrosis should undergo urgent debridement. However, the current conventional wisdom is that INP in clinically stable patients should be managed with antibiotics before surgery[16]. If the infected necrosis does not resolve, minimally invasive necrosectomy or open surgery is recommended once the necrosis is walled-off. Currently, a multidisciplinary consensus favors minimally invasive methods over open surgery to manage NP[16]. A randomized controlled trial clearly showed that endoscopic debridement is a better strategy than surgery[30]. Despite advances in surgical, radiologic, and endoscopic techniques, it is necessary to know that many patients with sterile pancreatic necrosis, and some patients with infected pancreatic necrosis, clinically improve sufficiently that they need no surgical intervention.

Table 4 Results of drainage without minimal invasive necrosectomy for necrotizing pancreatitis published mainly after 2000

Ref.	Year	Cases (*n*)	Type (Intend)	Sessions[1]	Success rate (%)	Morbidity (%)	Reoperation rate (%)	Mortality (%)	Remark
Sterile									
Baril et al[20]	2000	13	PCD		92.3		7.7	0.0	
Zerem et al[41]	2009	20	Conservative	1.5			15.0	0.0	RCT
		20	PCD	1.4	85.0		15.0	5.0	
Infected									
Freeny et al[52]	1998	34	PCD	3.3	47.0	26.0	53.0	12.0	
Baril et al[20]	2000	25	PCD	1.4	76.0	8.0	18.0	8.0	
Baron et al[44]	2002	38	TED + irrigation	2.0 (1-6)	79.0	NA	18.0	5.0	
Lee et al[19]	2007	18 WON	PCD + irrigation		83.3	11.0	16.7	5.6	Non-RCT; 32% PF
		5	TED		80.0		20.0	0.0	6% PF
Bruennler et al[42]	2008	80	PCD	(1-14)	43.0	29.0	25.0	34.0	1999-2004; 10 + PCN
Mortelé et al[43]	2009	35	PCD	3.3	49.0	11.0	37.0	17.0	
van Santvoort et al[18]	2011	130	PCD	NA	35.0	42.0	58.0	20.0	21 Dutch hospitals
Mukai et al[45]	2014	5 WON	SGTMD		100.0	0.0	0.0	0.0	
Sterile + infected									
Traverso et al[17]	2005	73	PCD	NA	79.0			11.0	
Chang et al[9]	2006	19 WON	MIS Lt. flank	1.0	84.2	10.5	5.2	15.8	80% INP
Babu et al[46]	2013	29	PCD; step-up			20.0		6.8	86% INP
Varadarajulu et al[47]	2011	48 WON	TED		52.1	10.4	35.4	6.5	
		12 WON	MTGT		91.7	0.0	0.0	0.0	

[1]Values are mean or range. INP: Infected necrotizing pancreatitis; MIS: Minimal invasive surgery; MTGT: Multiple transluminal gateway technique; NA: Not available; PCD: Percutaneous drainage; PCN: Percutaneous necrosectomy; PF: Pancreatic fistula; RCT: Randomized control study; SGTMD: Single transluminal gateway transcystic multiple drainage; TED: Transluminal endoscopic drainage; WON: Walled-off necrosis.

Minimally invasive necrosectomy

Although minimally invasive approaches are currently advocated, they still have some related morbidity and mortality[30,50]. Bausch et al[50] compared the outcomes of minimally invasive retroperitoneal necrosectomy (*n* = 14) and endoscopic transgastric necrosectomy (*n* = 18) with the outcomes of open necrosectomy (*n* = 30). Postoperative problems were ongoing sepsis (29%, 11%, and 73%, respectively) and bleeding that required intervention (21%, 17%, and 26%, respectively). A specific complication of endoscopic transgastric necrosectomy was gastric perforation into the peritoneal cavity during the procedure (28%), which required an immediate open pseudocystogastrostomy. A laparotomy was necessary in 21% of the patients after minimally invasive retroperitoneal necrosectomy and 28% after endoscopic transgastric necrosectomy because of specific complications or a persistent infection. The overall mortality rates were 21% and 6% after minimally invasive retroperitoneal and endoscopic transgastric necrosectomy, respectively, and significantly higher at 63% after open necrosectomy. Bausch et al[50] concluded that morbidity and mortality remained high in acute NP, and that surgery should be delayed as long as possible to reduce them. Minimally invasive procedures can preclude laparotomy, but they can also cause specific complications that require immediate or secondary open surgery.

Bausch et al[50] also found a lower mortality in the TEN group than in the minimally invasive retroperitoneal necrosectomy group, which is similar to what is shown in Table 3. Bakker et al[30] reported that the TEN group had significantly reduced proinflammatory responses, complications (20% *vs* 80%), new-onset multiple organ failure (0% *vs* 50%) and pancreatic fistulas (10% *vs* 70%)

compared to the video-assisted retroperitoneal drainage group. One gastric and one large intestine perforation occurred after video-assisted retroperitoneal drainage.

Ross et al[10] stated that "each treatment modality described for this application is a variation on a common theme-drainage of liquefied necrosis and debridement of necrotic tissue, either mechanically or by flushing and the passage of time". The key to complete evacuation of necrotic material is creating a large access opening to the cavity[33]. However, related complications such as bleeding, perforation, fistula, and embolism are inevitable[26,33,35,37,38]. TEN needs to be used with caution, ideally in an interdisciplinary approach and within clinical trials.

Drain first, but do it better

Earlier results of open or percutaneous drainage were not comparable with the open surgical necrosectomy with drainage procedure, and did not become the standard treatment of choice for INP.

In 2011, Windsor proposed "drain first, but do it better" for INP[51]. He pointed out that open necrosectomy is not the standard of care in many leading centers and is not an absolute requirement for INP. He concluded that PCD can be the only treatment for some patients with INP, which avoids an unnecessary necrosectomy. However, it has not yet been determined whether PCD is best used when infection is suspected or confirmed, nor has it been established when PCD can be delayed. Some interventional radiologists have long advocated primary PCD, but it has not been widely adopted[52]. This might soon change, however: 56% of patients-those with sterile NP and those with INP-did not require a surgical necrosectomy after PCD, according to one review[53]. The role

of PCD as the only treatment for INP needs additional evaluation so that it can be done better.

Ross et al[10] discussed a key point of "how to do it better", which is "the entry of the catheter into the collection was directed toward the dependent portion of the collection so that gravity could assist in drainage". The entry is therefore the left flank. Another key point is a large caliber drain and a big skin outlet. The author[9] used a 3-5 cm left-flank incision to enable the one-time insertion of a large-caliber sump drain directly through a liquefied route to the pancreatic head area where the drain was fixed on one side of the skin but the wound was kept open to enable the liquefied discharge to freely flow along the drain in case the drain lumen was obliterated by the debris. The open wound was pouched using a colostomy bag.

Garg et al[54] concluded that with medical management (conservative + PCD), surgery could be avoided in 76.6% patients. Other studies gave an even higher estimate (approximately 83%; Table 4). However, PCD alone failed in a significant proportion of patients[18,42,43,52] and a higher mortality rate has been reported[42].

TED and necrosectomy have been enormously pivotal in complementing the complete management of NP during this paradigm shift of intervention timing from prompt surgical debridement to delay until liquefaction. Promising results have been published. A step-up technique after a PCD failure is, it seems, the best way to "do it better." Repeated TEN[33] or TEN + TED showed an 80%-100% success rate (Tables 3 and 4); however TED is preferred because it avoids some complications of TEN.

Drainage with minimally invasive necrosectomy from transluminal endoscopy with or without a stent or from a trans-PCD sinus tract has its specific morbidities. The old important question of whether the necrosectomy is required[3,11] still remains unanswered. Can a "drain only" strategy further reduce these morbidities but maintain the same outcomes? With the progressive evolution of a multiple transluminal gateway technique for TED[47], single transluminal gateway transcystic multiple drainages[45], or dual modality drainage[10], albeit in only a few case series, allow 100% success when drainage without a necrosectomy is used to treat NP[10,43].

A transgastrostomy endoscopic procedure reported in 1993[55] (Table 3[25,28,32,34]) provides another feasible and easier access to further simplify the treatment. A double percutaneous endoscopic gastrostomy technique developed by Raczynski et al[25] was demonstrated as an inspiring tool. The author suggests a "one or two double-lumen transgastrostomy tube with jejunal arm method[32,56]" to offer jejunal feeding using a nasogastric tube, if needed, before the "delay until liquefaction" period, and an endoscopic or endoscopically assisted route for drainage later during the walled-off period. Creating a large access opening to the cavity to complete evacuation of necrotic material is essential[32]. One Foley tied with Penrose drains or two Foley drains keep the access open and offer irrigation.

Surgery: A last resort

Within the last decade, TEN and TED have probably replaced most surgical roles in the treatments of walled-off NP except for the disconnected pancreatic duct syndrome (DPDS)[17]. DPDS is characterized by evidence of a main pancreatic duct cutoff, an inability to access the upstream pancreatic duct during an endoscopic retrograde cholangiopancreatogram, and computed tomography evidence of viable pancreatic tissue upstream (toward the spleen), in association with a persistent non-healing pancreatic fistula or pancreatic-fluid collection, despite a course of conservative medical management[57]. DPDS is an increasingly recognized complication of severe AP and abdominal trauma, with reported prevalence rates that range from 10% to 31%. However, these figures are most representative of highly select populations of severe AP in tertiary hospitals; the prevalence in all cases of AP remains unknown[58]. Although surgical management had been the consensus[59], there have been reports of success with initial endoscopic treatment[57]; 19/26 patients showed long-term improvement, seven required surgery after treatment failed, the other five underwent immediate surgery: mortality was 0%. Sarkaria et al[32] included four DPDS patients for a series in which they used esophageal stents to treat walled-off pancreatic necrosis. Complete resolution was achieved in 15/17 patients (88%). Two patients not specified as having DPDS required surgical intervention after endoscopic treatment failed. At least two of their patients did not need surgical rescue for DPDS. More effort should be focused on DPDS in the era of transluminal endoscopic treatment.

Necrosectomy: Obsolete

The answer to "will necrosectomy be obsolete?" is now much more positive. A paradigm-shift from a surgical to a non-surgical approach, or to drainage as proposed by Windsor[51], whether the necrosis is infected or sterile, is waiting for additional randomized studies. Although not significantly different, endocrine (diabetes) and exocrine insufficiency were lower in the endoscopic drainage group than in the surgery group[60]. Unnecessary necrosectomy procedures for the risky disease of NP should be prevented[5,17] and surgical management should be used as a last resort.

CONCLUSION

With the recent successful outcomes of pure endoscopic and complementary endoscopic treatments for failed PCD, it is clear that drainage without a necrosectomy is feasible and should be the first choice of treatment for symptomatic sterile or infected walled-off NP and peripancreatic fluid collection. A paradigm shift from necrosectomy to drainage for the treating NP should be considered to eliminate potential complications.

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2014 ADVANCES IN PANCREATITIS

Immunomodulatory therapies for acute pancreatitis

Jing Li, Wen-Juan Yang, Lu-Ming Huang, Cheng-Wei Tang

Jing Li, Lu-Ming Huang, Cheng-Wei Tang, Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Jing Li, Wen-Juan Yang, Cheng-Wei Tang, Division of Peptides Related with Human Diseases, Key Laboratory of Biotherapy of Human Diseases, Ministry of Education, Chengdu 610041, Sichuan Province, China
Author contributions: All the authors contributed equally to the manuscript.
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Correspondence to: Cheng-Wei Tang, MD, PhD, Department of Gastroenterology, West China Hospital, Sichuan University, No. 37 Guoxuexiang, Wuhou District, Chengdu 610041, Sichuan Province, China. shcqcdmed@163.com
Telephone: +86-28-85422383 Fax: +86-28-85582944
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Abstract

It is currently difficult for conventional treatments of acute pancreatitis (AP), which primarily consist of anti-inflammatory therapies, to prevent the progression of AP or to improve its outcome. This may be because the occurrence and progression of AP, which involves various inflammatory cells and cytokines, includes a series of complex immune events. Considering the complex immune system alterations during the course of AP, it is necessary to monitor the indicators related to immune cells and inflammatory mediators and to develop more individualized interventions for AP patients using immunomodulatory therapy. This review discusses the recent advances in immunomodulatory therapies. It has been suggested that overactive inflammatory responses should be inhibited and excessive immunosuppression should be avoided in the early stages of AP. The optimal duration of anti-inflammatory therapy may be shorter than previously expected (< 24 h), and appropriate immunostimulatory therapies should be administered during the period from the 3rd d to the 14th d in the course of AP. A combination therapy of anti-inflammatory and immune-stimulating drugs would hopefully constitute an alternative to anti-inflammatory drug monotherapy. Additionally, the detection of the genotypes of critical inflammatory mediators may be useful for screening populations of AP patients at high risk of severe infections to enable the administration of early interventions to improve their prognosis.

Core tip: In light of the complex immune system alterations that occur in acute pancreatitis (AP), it is necessary to develop more individualized interventions for AP patients by using immunomodulatory therapy instead of inflammatory drug monotherapy. We first suggest how we could monitor the immune status of these patients and identify optimal treatment methods. We also demonstrate for the first time that the detection of the genotypes of critical inflammatory mediators may be useful for screening populations of AP patients at high risk of severe infections.

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INTRODUCTION

Acute pancreatitis (AP) is a common acute abdominal disease. Despite advances in treatment, the mortality rate of severe acute pancreatitis (SAP) remains as high as 10%-30%, and multiple organ dysfunction is the main

cause of death[1-3].

Currently, conventional treatments for AP include fluid infusion, inhibition of pancreatic secretion and organ support. However, fluid resuscitation cannot prevent pancreatic necrosis[4,5]. In addition, the inhibition of pancreatic secretions has always been considered one of the most important strategies for treating AP. Research has shown that cell apoptosis or necrosis occurs in AP patients, that the zymogen granules of acinar cells decrease in number and that pancreatic exocrine functions are inhibited[6]. Under such conditions, the inhibition of exocrine pancreatic function cannot prevent the progression of AP or improve its outcomes[6,7]. Because AP consists of chemical inflammation, the prophylactic use of antibiotics cannot lower the incidence of infection in a necrotic pancreas or the mortality of AP[8-10]. Although organ support and symptomatic treatment may help patients survive multiple organ failure[11,12], there is still a lack of effective treatment for AP.

Until now, conventional treatments for AP have mainly been anti-inflammatory therapies. However, their effects have not proven to be as satisfactory as expected[4]. In fact, numerous studies have shown that a variety of inflammatory cells and cytokines are involved in the occurrence and progression of AP, which comprises a series of complex immune events[13]. A thorough understanding of the AP immune response and its mechanism can aid in the development of better AP treatment strategies.

AP IMMUNE RESPONSE (INFLAMMATORY RESPONSE AND IMMUNE SUPPRESSION)

The onset of AP generally includes the following immunological stages: systemic inflammatory response syndrome (SIRS), compensatory anti-inflammatory response syndrome (CARS) and mixed anti-inflammatory response syndrome (MARS)[4,14-16]. In light of the complex immune system alterations that occur during the different phases of AP, a more individualized approach to AP, such as the use of immunomodulatory therapy, may be beneficial.

SIRS

Following stimulation by various pathogenic factors, trypsin in the pancreatic acinar cells is prematurely activated due to neutrophil involvement[17]. This process is closely related to the following pathophysiological processes: high pancreatic duct pressure, the flow of Ca^{2+} into the pancreatic acinar cells and the activation of transcription factors, such as nuclear factor-κB (NF-κB)[6,12,18]. NF-κB is a core molecule of the innate immune response. The amplification of inflammatory signals generated by NF-κB is able to produce a large number of inflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and monocyte chemotactic protein (MCP)-1[19]. In the presence of these cytokines and chemokines, more neutrophils and lym-

phocytes gather in the pancreas and intestinal tracts, thus amplifying the inflammatory response[20]. When these pro-inflammatory cytokines are released into the blood, the inflammation is no longer confined to the pancreas, and, consequently, SIRS develops. The activated neutrophils and monocytes are then able to release proteases and oxygen free radicals. These inflammatory mediators can then cause damage to the vascular endothelial cells, increases in vascular endothelial permeability and the accumulation of a large amount of fluid in the tissue. As a result of these processes and other microvascular dysfunctions, tissue hypoxia and significant organ dysfunction develop[16].

In addition, intestinal ischemia-reperfusion during AP can significantly upregulate the expression of pattern recognition receptors, such as toll-like receptors (TLRs), in the intestinal mucosa, potentially leading to an overactive intestinal mucosal immune response and the rapid progression of AP[21]. Animal experiments have shown that TLR4-deficient mice rarely develop SAP[22]. Furthermore, TLR4 gene polymorphisms have been found to be associated with susceptibility to severe infections in AP patients[23].

CARS

With the release of pro-inflammatory cytokines, anti-inflammatory cytokines are produced in the body. The outcome of this disease depends on the balance between the inflammatory and anti-inflammatory responses. When the anti-inflammatory response is strong enough, patients may recover. On the contrary, when the anti-inflammatory response is not sufficiently strong, an excessive inflammatory response can lead to early organ dysfunction and SAP. During periods of overactive compensatory anti-inflammatory responses, the following changes may occur in AP: anergy in lymphocytes, thymus and spleen atrophy and a decrease in the number of peripheral lymphocytes (mainly the T lymphocytes), which could be associated with a depletion of lymphocytes or with lymphocyte apoptosis[13,24-27]. The abovementioned changes during immunosuppression are closely related to infection in the late stage of SAP[28].

In addition to lymphocyte-related defective defense systems in AP, mononuclear cell dysfunction may occur, which is characterized by a significant decrease in the expression of human leukocyte antigen (HLA-DR) and the synthesis of pro-inflammatory cytokines (*e.g.*, TNF-α)[16]. The expression of low-density HLA-DR in mononuclear cells indicates an impaired antigen-presenting function. Mentula *et al*[29,30] reported that reduced expression of HLA-DR in monocytes in early SAP most likely predicted the occurrence of a secondary infection and fatal complications in AP. HLA-DR expression can be detected by flow cytometry within 30-60 min; therefore, it can be used as a routine test to identify the immune status of AP patients and to predict the outcomes of AP[13].

Furthermore, intestinal immune function can be impaired in early AP, manifesting as damage to the integrity
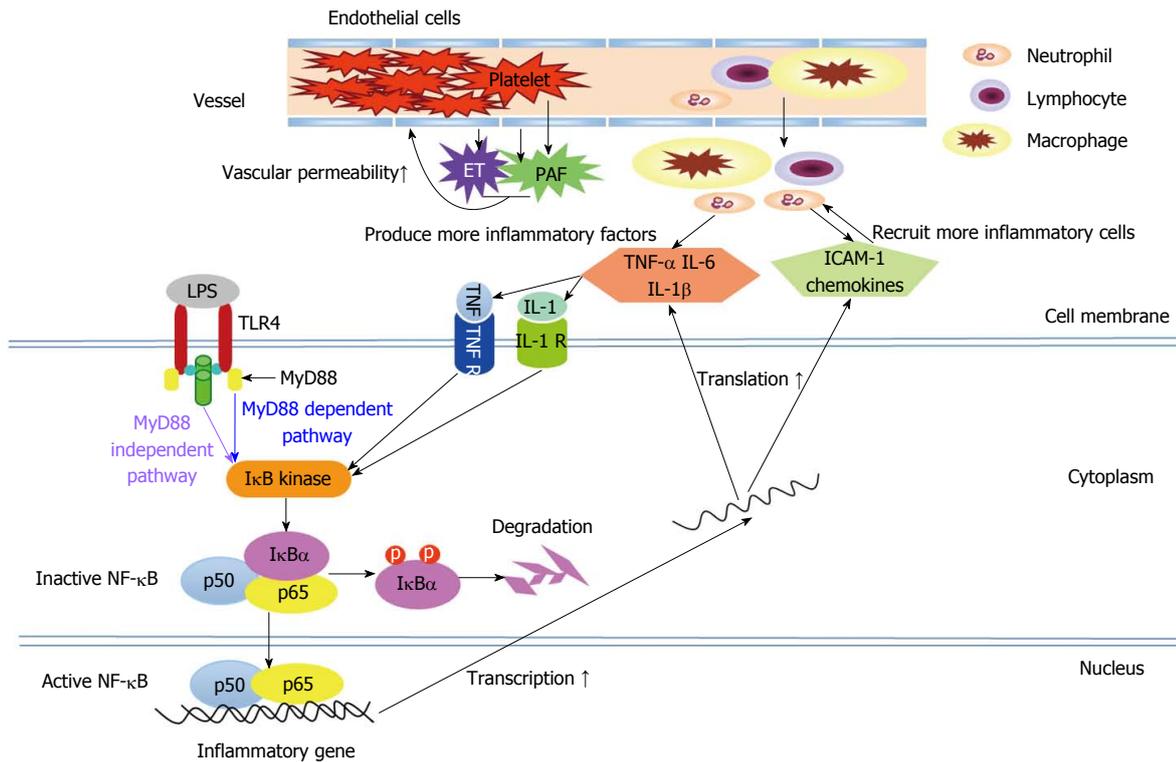
Figure 1 Schematic presentation of the pathways involved in the inflammatory response during acute pancreatitis. Following lipopolysaccharide (LPS) stimulation, the inhibitors of κB (IκB) kinase are activated *via* the toll-like receptor 4 (TLR4)-myeloid differentiation factor 88 (MyD88)-dependent (blue) or -independent pathway (purple). Thereafter, IκBα is rapidly phosphorylated by IκB kinase and then degraded. This process allows nuclear factor-κB (NF-κB) to translocate into the nucleus and to increase the transcription of several important inflammatory genes, such as the genes encoding tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), adhesion molecules and chemokines. The up-regulation of these inflammatory mediators, in turn, either leads to further IκB kinase activation or mediates the migration of the inflammatory cells to the site of inflammation, thus amplifying the inflammatory response. Meanwhile, platelet-activating factor (released from active macrophages, endothelial cells or platelets) and endothelins (released from endothelial cells) are involved in the increased vascular permeability and extravasation of inflammatory cells. TNF R: Tumor necrosis factor receptor; IL-1 R: Interleukin-1 receptor; ICAM-1: Intercellularadhesionmolecule-1; PAF: Platelet-activating factor; ET: Endothelin.

of intestinal mucosal barriers, decreased levels of immune cells and the absence of secretory immunoglobulin A (sIgA) in the intestine. These events may in turn cause translocations of intestinal bacteria and endotoxins, as well as infectious complications[31].

MARS

SIRS, MARS and CARS are traditionally thought to appear successively in AP. MARS is regarded as a transient dynamic balance in the transition from SIRS to CARS. However, increasing evidence has revealed that there is no distinct boundary between SIRS and CARS and that CARS also occurs in the early stages of sepsis or SAP[14,28]. Therefore, MARS cannot be a transition period from SIRS to CARS; instead, it may constitute an independent reaction in tandem with an excessive inflammatory response and immune suppression in early SAP[4,14-16].

ANTI-INFLAMMATORY THERAPIES

In AP, the overactivity of pro-inflammatory cells and factors may aggravate local inflammation in the pancreas, facilitating the development of SIRS. Anti-inflammatory factors will not work effectively when their levels are be-

low those of the pro-inflammatory factors[32,33]. Theoretically, the modulation of immunocytes and inflammatory mediators may be able to prevent overactive immune reactions and to alleviate inflammatory injuries; this hypothesis has been gradually confirmed by accumulating animal experiments and clinical trials over the past 20 years. Blocking these inflammatory pathways (Figure 1), moderately and at the appropriate time, may be an effective treatment for AP. However, the inflammatory reaction involves numerous factors and cells. Thus, treatments targeting only certain factors or cells may be unable to terminate the entire overactive inflammatory reaction on their own.

NF-κB

NF-κB, a vital transcription factor, is activated early in the course of AP and regulates the transcription of many genes involved in inflammatory responses, such as TNF-α, IL-1β and IL-6[18] (Figure 1). It has been found that the inhibition of NF-κB activity with amobarbital, a NF-κB essential modifier binding domain peptide, or pyrrolidine dithiocarbamate (NF-κB activity inhibitor) attenuated AP-associated injuries in the pancreas and lungs. Currently, these results are only based on animal studies[34-36].

Table 1 Clinical trials of lexipafant in acute pancreatitis

Ref.	Study design	Severity of AP	No.	Interval	Dosage and administration	Major variables	Outcomes
Kingsnorth et al[74], 1995	Multi-center double-blind RCT	Mix	83	< 48 h	60 mg/d, i.v., × 3 d	Organ failure OFS IL6, IL8	Positive
McKay et al[75], 1997	Multi-center double-blind RCT	APACHEII > 5, Glasgowscore ≥ 3, and C-reactive protein ≥ 120 mg/L	50	< 72 h	100 mg/d, i.v., × 7 d	OFS Systemic complications Mortality	Positive
Johnson et al[76], 2001	Multi-center double-blind RCT	APACHEII > 6	290	< 72 h	100 mg/d, i.v., × 7 d	Systemic sepsis Pseudocysts New organ failure. Mortality	Positive in systemic sepsis and pseudocysts

AP: Acute pancreatitis; No.: Number of patients; Interval: Time interval between AP onset and the initiation of octreotide treatment; RCT: Randomized controlled trial; Mix: Mixture of APACHEII < 8 and APACHEII ≥ 8 acute pancreatitis patients; i.v.: Intravenous infusion; OFS: Organ failure score; IL: Interleukin; APACHE: Acute Physiology and Chronic Health Evaluation scale.

TNF-α

TNF-α is a monocyte-derived pro-inflammatory cytokine. In AP, TNF-α was found to contribute to pancreatic acinar cell death and to the expression of other pro-inflammatory factors, such as IL-1 and IL-6[37,38] (Figure 1). Animal studies have shown that TNF-α blockade with anti-TNF-α antibodies or soluble TNF-α receptors was able to decrease the severity of AP and reduce the associated mortality rate by prohibiting the effect of TNF-α[39-43]. In addition, several case reports have demonstrated that infliximab (monoclonal anti-TNF-α antibody) was effective in AP-complicated acute Kawasaki or active Crohn's disease and had a preventive effect on AP in a patient with recurrent acute exacerbation of chronic pancreatitis[44,45].

IL

Various pro-inflammatory interleukins, such as IL-1β, IL-6, IL-8 and IL-18, are involved in the inflammatory responses associated with AP[46-51]. Animal studies have suggested that the inhibition of pro-inflammatory interleukins with antibodies (e.g., anti-IL-6 receptor antibody or anti-IL-8 antibody), receptor antagonists (IL-1 receptor antagonist) or biosynthesis inhibitors (IL-1-converting enzyme inhibitor) ameliorated pancreatic and lung injuries in AP and reduced the associated mortality rate[46,52-56].

During the overactive inflammatory reactions of AP, the anti-inflammatory cytokine IL-10 is unable to inhibit the inflammatory reactions effectively, as its serum level is far below that of the pro-inflammatory factors, such as TNF-α[32,33]. In animal experiments associated with AP, exogenous IL-10 or supplementation with its effective fragment (IT9302), IL-10 gene transfer and insulin-like growth factor administration were each reported to result in the downregulation of serum TNF-α levels, the alleviation of pancreatic injury and a decrease in death rates by increasing the circulating levels of IL-10 or its effective fragment[57-63]. In clinical trials, there were conflicting results regarding the ability of IL-10 to prevent post-endoscopic retrograde cholangiopancreatography

pancreatitis[64-66]. Different inclusion criteria and follow-up times may be responsible for the controversy. Nevertheless, persistent elevation in circulating IL-10 levels has been found to be one of the causes of immunosuppression in some AP patients[67].

Although the effectiveness of anti-inflammatory therapies targeting IL has been confirmed by many animal experiments, only treatment with IL-10 has been investigated through clinical trials. Larger randomized clinical trials (RCTs) are required to assess the safety and efficacy of these therapies.

Platelet activating factor

Platelet activating factor (PAF), a biologically active phospholipid that is released from macrophages, endothelial cells and platelets, has been shown to induce neutrophil-platelet aggregation and to increase vascular permeability[68] (Figure 1). Lexipafant, a PAF antagonist, is able to inhibit the inflammatory response and to ameliorate the severity of pancreatitis-associated intestinal and lung damage in experimental models of AP[69-73]. Several large-scale RCTs have also indicated that lexipafant led to significant decreases in mortality and the incidence of systemic complications of AP[74-76] (Table 1). The recommended administration of lexipafant is intravenous infusion at a dose of 100 mg/d for 7 d within 72 h after AP onset[74,75]. However, a phase III clinical trial indicated that lexipafant had no effect on new organ failure or death rates in AP[76].

Endothelin

Endothelins (ETs: including ET-1, ET-2 and ET-3) and their receptors (ETA and ETB) mediate the vasoconstriction response and maintain vascular tension[77]. It has been found that elevated plasma endothelin-1 levels are associated with low perfusion and pancreatic necrosis in AP[78,79]. Currently, it remains controversial whether ET receptor antagonists have beneficial effects on pancreatic perfusion and the mortality of AP[80-86]. However, studies have involved only animal experiments, and no relevant

Table 2 Clinical trials of octreotide in acute pancreatitis

Ref.	Study design	Severity of AP	No.	interval	Dosage and administration	Major variables	Outcomes
Wang *et al*[89], 2013	Prospective RCT	P-SAP; SAP	P-SAP: 236 SAP: 136	< 48 h	50 μg/h, continued *i.v.*, × 3 d + 25 μg/h, continued *i.v.*, × 4 d, or 25 μg/h, continued *i.v.*, × 7 d	APACHE II, SIRS score, and MOF score Local complication IL6, TNF-α	Positive at higher dosage
Yang *et al*[90], 2012	Multi-center RCT	MAP	161	< 48 h	50 μg/h, continued *i.v.*, × 3 d	APACHE II and MOF score Local complication IL6, TNF-α	Positive
Nikou *et al*[91], 2004	Prospective RCT	MAP	36	< 12 h	200 or 500 μg, *i.h.*, 3 times/d × 5 d	IL-6, C-reactive protein	Positive IL-6 outcome at higher dosages
Beechey-Newman[93], 1993	Prospective case-control study	MAP	19	N	250 μg, *i.h.*, then 0.5 μg/kg per hour, continued *i.v.*, × 10 d	Biochemical and physiological parameters	Positive in serum calcium, albumin, hematocrit, hemoglobin, PaO2
Binder *et al*[94], 1994	Prospective trial	Mix	8	N	100, 200 or 500 μg, *i.h.*, 3 times/d × 10 d	Complications	Positive at two higher dosages
Paran *et al*[95], 1995	Multi-center RCT	MAP	38	N	100 μg, *i.h.*, 3 times/d × 14 d	Organ dysfunction Local complications Length of hospital stay Mortality	Positive in organ dysfunction and length of hospital stay
Fiedler *et al*[96], 1996	Prospective case-control study	SAP	39	N	100 μg, *i.v.*, 3 times/d × 10 d	Organ dysfunction Mortality	Positive
McKay *et al*[97], 1997	Multi-center RCT	Mix	58	N	40 μg/h, continued, *i.v.*, × 5 d	Complications Mortality	Negative
Karakoyunlar *et al*[98], 1999	Prospective controlled study	Mix	43	N	250 μg/h, continued *i.v.*, × 2 d	Biochemical, physiological and radiological changes Mortality	Positive outcomes for serum amylase levels, pancreatic edema and earlier return to oral intake
Paran *et al*[99], 2000	Case-controlled study	Mix	50	N	100 μg, *i.h.*, 3 times/d, × 14 d	Organ dysfunction Local complications Length of hospital stay Mortality	Positive in organ dysfunction, length of hospital stay and mortality
Nikou *et al*[100], 2001	Prospective RCT	Mix	120	N	100, 200 or 300 μg, *i.h.*, 3 times/d, × 7 d	Duration of pain Organ dysfunction Local complications	Little benefit only at two higher dosages

AP: Acute pancreatitis; No.: Number of patients; Interval: the time interval between AP onset and the initiation of octreotide treatment; RCT: Randomized controlled trial; MAP: Mild acute pancreas; SAP: Severe acute pancreas; Mix: Mixture of MAP and SAP; P-SAP: Predicted SAP; N: Not mentioned; *i.h.*: Subcutaneous infusion; *i.v.*: Intravenous infusion; APACHE: Acute Physiology and Chronic Health Evaluation scale; SIRS: Systemic inflammatory response syndrome scale; MOF: Multiple organ failurescale; IL: Interleukin; TNF-α: Tumor necrosis factor-α; PaO2: Arterial oxygen partial pressure.

clinical studies have been completed to date.

Somatostatin

Somatostatin (SST), a multifunctional neuropeptide, is mainly released from sensory nerve endings and gastro-intestinal neuroendocrine cells. Accumulating evidence has suggested that SST causes a significant anti-inflammatory effect in AP, in addition to its potential roles in inhibiting exocrine pancreatic function and regulating the tone of the sphincter of Oddi[6]. Our previous animal experiments demonstrated that SST could relieve inflammatory injuries by blocking the TLR4-myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (Figure 1), as well as by inhibiting the activity of intestinal mucosal mast cells[21,87,88]. Over the past two years, our clinical studies have indicated that plasma SST levels decreased within 48 h after AP onset, along with increased plasma levels of IL-6 and TNF-α[89,90]. Thus, an early replacement of exogenous SST or its analogue,

octreotide, may be beneficial for patients with AP. Our recent prospective RCTs have shown that octreotide administration attenuated SAP to some extent and prevented the development of SAP in obese patients and other patients with predicted SAP by raising their plasma SST levels and decreasing their circulating levels of TNF-α and IL-6[89-91]. However, the effect of SST or octreotide on AP remains controversial[92-100]. Different timings, doses or durations of octreotide administration may contribute to these disputed results (Table 2). More research is needed to fully understand the roles of SST and octreotide in AP and to identify the optimal treatment timing and dosage.

Immune cells and related factors

In AP, immune cells (macrophages, monocytes, neutrophils and lymphocytes) migrate to the sites of inflammation and release pro-inflammatory cytokines and chemokines with the aid of chemokines and adhesion

molecules, which recruit even more immune cells, aggravating the inflammation[13]. There is increasing evidence that inhibiting the activation and migration of immune cells may have a therapeutic effect on excessive inflammatory responses[20,101-103].

Macrophages are one of the major classes of immune cells involved in the pathogenesis of AP. They mainly present as M1 macrophages to release pro-inflammatory cytokines and to aggravate pancreatic and systemic inflammation[101,102]. In an *in vitro* study, IL-4 and IL-13 were able to convert M1 macrophages into M2 macrophages, which have an anti-inflammatory role. However, these cytokines failed to show similar results *in vivo*[101]. In addition, hemin and gadolinium chloride were able to attenuate the inflammation and AP-associated organ injuries in rats by inhibiting the pro-inflammatory effects of macrophages[102,103]. Although there is no related clinical trial, the results from the above animal studies may provide a new direction for the development of immunomodulatory therapy for AP[102,103].

Chemokines and their receptors contribute to the migration of leukocytes to areas of injury and the development of inflammation[104]. Chemokines can be broadly divided into CXC and CC subgroups. In the CXC subgroup, the first two cysteine residues (C) out of four are separated by another amino acid (X), whereas in the CC subgroup, the first two cysteine residues are adjacent[104,105]. Many animal experiments on AP have revealed that the blockage of chemokine synthesis (monocyte chemotactic protein-1 or fractalkine) or neutralization of chemokines with antibodies (anti-cytokine-induced neutrophil chemoattractant antibodies or anti-CC receptor 5 ligand antibodies) was able to relieve inflammatory reactions, increasing the survival rates[104-109]. In addition, the inhibition of combined chemokines (CXC or CC) and their receptors was also found to have a similar therapeutic effect on AP[110-112]. However, those results have not been confirmed by clinical trials.

Adhesion molecules are glycoprotein molecules that are located on the cell surface and are involved in binding to other cells or the extracellular matrix. Intercellular adhesion molecule-1 (ICAM-1) is one of the adhesion molecules expressed on endothelial cells; it mediates the adhesion and migration of immune cells, facilitates leukocyte infiltration and exacerbates systemic inflammatory reactions[3,83,113-118]. Studies using animal models of AP have shown that ICAM-1 antibodies attenuate inflammatory cell infiltration and pancreatic and lung injuries[83,114-116,118]. Unfortunately, there are no relevant clinical research data currently available.

IMMUNOSTIMULATORY THERAPIES

In the late stages of AP, a decreased number of lymphocytes can lead to impaired cellular and humoral immune function, including the inability to release cytokines and reduced ratios of CD4^{+}/CD8^{+} T cells and Th1/Th2 helper T cells[28,119]. These changes, together with mono-

cyte dysfunction, will most likely render the host susceptible to infection and death due to pathogenic invasion. Therefore, immunostimulation targeting CD4^{+} cells, Th1 cells and monocytes may be effective for treating or preventing infectious complications of SAP. Monitoring immune cells and cytokines could be useful for predicting the prognosis of AP[119,120].

Restoring the balance in number and function of immune cells

It has been found that a reduction in the circulating CD4^{+} T cell levels and the CD4^{+}/CD8^{+} ratio may be partly responsible for the development of immunosuppression in SAP[121]. The application of thymosin alpha 1 showed a protective effect against SAP in rats, improving their survival rate by restoring serum CD4^{+} T cell levels and the CD4^{+}/CD8^{+} ratio[122]. Moreover, an imbalance between Th1 and Th2 cells was also found to be associated with the pathogenesis of immunosuppression in SAP, which included deficiencies in the number and function of Th1 cells and a large number and hyperfunctionality of Th2 cells[123,124]. Many studies have revealed that the granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interferon-γ (IFN-γ) have been able to restore the balance between Th1 and Th2 to some extent, based on *in vitro* and *in vivo* experiments associated with AP[122,125,126]. However, clinical studies supporting these findings remain sparse.

Additionally, monocyte dysfunction may result in AP-associated immunosuppression, including impaired antigen presentation capacity (marked by reduced HLA-DR expression) and insufficient synthesis of pro-inflammatory cytokines[127]. The administration of IFN-γ or GM-CSF was reported to raise the HLA-DR expression levels on monocytes and to enhance their capacity for TNF-α production in septic patients[128,129]. The only related RCT in the past five years also showed that the subcutaneous injection of GM-CSF (4 mg/kg per day) for 8 d was safe and effective for restoring monocytic immunocompetence and shortening the course of sepsis-associated immunosuppression[130]. GM-CSF or IFN-γ administration *in vitro* was able to upregulate HLA-DR expression and the TNF-α production of monocytes from SAP patients[127]. It has been suggested that combination therapy of GM-CSF and IFN-γ is able to completely reverse monocyte dysfunction[127]. To date, GM-CSF and IFN-γ have been widely used in the treatment of sepsis. More research is needed before GM-CSF and IFN-γ can be used in patients with AP-associated immunosuppression.

Restoring intestinal immune function

Intestinal immunosuppression may occur in early SAP (within 24 h after AP onset) and is characterized by a significant decrease in sIgA secretion and in the number of CD4^{+} T lymphocytes in the intestinal mucosa, which is one of the most important causes for bacterial and endotoxin translocation[31]. Restoring intestinal immune function as early as possible may represent a promising

treatment to prevent infectious complications in SAP. In animal experiments on SAP, oral supplementation with arginine, glutamine and probiotics increased the number of CD4^{+} T lymphocytes and sIgA levels in the intestine and circulation[31,131]. Furthermore, clinical trials have reached similar conclusions. Early enteral nutrition (within 48 h after admission) has been suggested to cause an increase in serum IgG levels and HLA-DR expression in T lymphocytes, thus reducing the incidence of multiple organ dysfunction syndrome, SIRS and pancreatic infection[152].

For patients with AP-associated immunosuppression, proper immunostimulation may alleviate the disease and prevent serious complications[130,132]. However, those results are mainly based on animal or *in vitro* studies. More large-scale RCTs are needed to evaluate the safety and efficacy of immunostimulatory therapies for AP-associated immunosuppression.

CLINICAL STRATEGIES FOR IMMUNOMODULATION IN AP

As mentioned above, different treatment regimens should be administered to AP patients according to their different immune statuses. However, it is currently unclear how the immune status of patients can be monitored to identify the optimal treatment timing. Genetic testing for gene polymorphisms in certain inflammatory mediators can be used to screen high-risk AP patients for severe infection, thus preventing severe infections[23,133,134].

Immune state monitoring of AP patients and interventional window

Clinical trials have shown that peripheral blood lymphocyte levels are significantly reduced to approximately 67% of the lower limit of normal within 24-72 h after a SAP attack[28,135]. This reduction may be associated with the depletion of lymphocytes, lymphocyte apoptosis or gut associated lymphocyte homing[13,24-27,136]. During the same period, peripheral blood CD4^{+} T cell levels, the ratio of CD4^{+}/CD8^{+} T cells and the ratio of Th1/Th2 helper T cells all significantly decreased[27,28,123,124,137]. Moreover, the antigen-presenting function of monocytes was also impaired (characterized by low HLA-DR expression) within 24-72 h after SAP onset[27,30,138]. It has been reported that a continuous decline of the above indicators from the 7th to the 14th d in the course of AP could predict a higher risk of infectious complications[27,28,30,123,124,138-140].

Many of the findings in the literature have revealed that the serum levels of pro-inflammatory cytokines (*e.g.*, IL-8, TNF-α, IL-6, IL-2, IL-12 and IFN-γ)[67,123,124,137,138,141-143] and anti-inflammatory cytokines (*e.g.*, IL-10 and IL-4)[67,123,124,138,141] were significantly elevated within 12-72 h after a SAP attack; thereafter, the shared pattern diverged. Retrospective studies in the past two years have suggested that the serum TNF-α, IL-6, IL-12 and IFN-γ levels gradually decreased in the 3rd-5th d after the onset of SAP[67,137]. In addition, the serum IL-10 and IL-4 levels of SAP patients with infections showed a continuous upward trend

from the 3rd d to the 14th d after a SAP attack, whereas the anti-inflammatory cytokine levels of patients without infections declined continuously[67]. Despite certain shortcomings, serum cytokine levels remain one of the most important rapid indicators of a patient's immune status[120].

Monitoring the levels of immune cells and cytokines could enable the early detection of the immune suppression state over the course of SAP, and their appropriate modulation could be expected to reduce the incidence of infection and death.

In addition, clinical research completed during the past year showed that the overexpression of the factor-associated suicide (Fas) ligand on T lymphocytes in SAP patients resulted in the excessive apoptosis of T lymphocytes and a sharp drop in CD4^{+} T cells, thus inducing immune suppression and sepsis[27]. Fas expression on T lymphocytes in SAP patients was transiently reduced within 48 h after AP onset but continuously increased within 8 d thereafter[124]. Therefore, for AP patients with continuously high expression of Fas for 10 d after AP onset, appropriate immune stimulation may improve the immune suppression state.

The interventional window for AP mentioned in the previous studies refers to the timing of anti-inflammatory therapy. Many researchers have found that this window usually arose between 12-18 h and 2-3 d after the onset of AP[16]. However, accumulated evidence has indicated that the timing for both anti-inflammatory and immune stimulation therapies should be considered. Thus, according to the above data, the duration of anti-inflammatory therapy may be shorter than expected (< 24 h), as immunosuppression can occur within 24 h after SAP onset[27,28,30,67,123,124,137-140], whereas appropriate immune stimulation therapy should be administered during the period between the 3rd and 14th d in the course of AP[27,28,30,67,123,124,137-140]. Nevertheless, the optimal interventional window of AP needs to be defined by further prospective studies.

Combination of anti-inflammatory and immune-stimulating therapies

Many factors are involved in the pathogenesis and progression of AP. The studies available on monotherapies for a certain factor did not yield the desired results. Thus, increasingly more investigators have begun to focus on multi-drug combination therapies for AP. Their studies have confirmed that a multi-drug combination is more effective than monotherapy. These therapies have comprised either the combination of various types of anti-inflammatory drugs[144,145] or a combined drug treatment for immunosuppression[127]. Currently, there are no published reports on the combination of anti-inflammatory and immune-stimulating therapies for AP, although this topic holds great promise as a new approach to AP immunotherapy.

AP populations at high risk of severe infections

Genetic polymorphisms of certain inflammatory mediators were found to be closely related to infections in AP

patients. It has been reported that AP patients with TLR4 Asp299Gly mutations were prone to necrosis and infection of the pancreas[23] and that SAP patients carrying the IL-10-1082G[133], TNF2 and TNFB2 alleles[134] were highly susceptible to septic shock. Therefore, the detection of these genotypes could be helpful in screening high-risk AP populations for severe infections. These findings suggest that we may be able to develop more successful interventions for those patients in the future, potentially preventing more severe infections.

CONCLUSION

In summary, immunomodulatory therapy will hopefully improve the outcomes of AP. In the course of AP, it is necessary to monitor the indicators related to immune cells and inflammatory mediators and to develop more successful and individualized interventions for AP patients using immunomodulatory therapies. During the early stages of AP, treatment should include the inhibition of the overactive inflammatory responses while avoiding excessive immunosuppression to maintain normal immune function and to reduce the incidence of infectious complications and organ failure. Furthermore, the duration of anti-inflammatory therapies may be shorter than previously expected (< 24 h), whereas appropriate immune stimulation therapy should be administered between the 3rd and 14th d of the AP disease course. The combined therapy of anti-inflammatory and immune-stimulating drugs may represent an alternative to anti-inflammatory drug monotherapies. Finally, the detection of the genotypes of critical inflammatory mediators would facilitate early interventions in AP populations at high-risk for severe infections to improve their prognosis.

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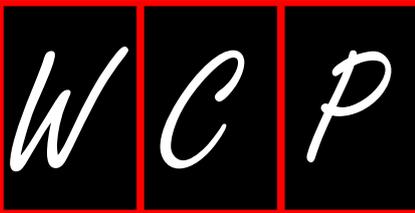
WJG 20th Anniversary Special Issues (18): Pancreatitis

Genetic and phenotypic heterogeneity in tropical calcific pancreatitis

Sumit Paliwal, Seema Bhaskar, Giriraj R Chandak

Sumit Paliwal, Seema Bhaskar, Giriraj R Chandak, CSIR-Centre for Cellular and Molecular Biology (CSIR-CCMB), Hyderabad 500007, India

Giriraj R Chandak, Adjunct Group Leader, Genome Institute of Singapore, Biopolis 138672, Singapore

Author contributions: Paliwal S wrote the first draft of the review with substantial contributions from Bhaskar S; Chandak GR designed and planned the structure of the review, provided critical suggestions and finalized the contents; all authors have seen the final version and approved it.

Correspondence to: Giriraj R Chandak, MD, PhD, CSIR-Centre for Cellular and Molecular Biology (CSIR-CCMB), Uppal Road, Habsiguda, Hyderabad 500007,
India. chandakgrc@ccmb.res.in
Telephone: +91-40-27192748 Fax: +91-40-27160591
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Abstract

Tropical calcific pancreatitis (TCP) is a form of chronic non-alcoholic pancreatitis initially reported in the developing parts of the tropical world. The clinical phenotype of TCP has undergone marked changes since its first description in 1968. The disease is now seen in relatively older people with less severe symptoms. In addition, there are varying reports on the proportion of cases presenting with imaging abnormalities like calcification, ductal dilation, and glandular atrophy. Significant progress has also been made in understanding the etiopathology of TCP. The role of malnutrition and cassava toxicity in its pathogenesis is disproven and few studies have focused on the role of micronutrient deficiency and oxidative stress in the etiopathogenesis of TCP. Emerging evidence support an important role for genetic risk factors in TCP. Several studies have shown that, rather than mutations in trypsinogens, variants in serine protease inhibitor kazal type 1, cathepsin B, chymotrypsin C, cystic fibrosis transmembrane regula- tor, and carboxypeptidase A1, predict risk of TCP. These studies also provided evidence of mutational heterogeneity between TCP and chronic pancreatitis in Western populations. The current review summarizes recent advances that have implications in the understanding of the pathophysiology and thus, heterogeneity in genotype-phenotype correlations in TCP.

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Key words: Chronic pancreatitis; Tropical calcific pancreatitis; Fibrocalculous pancreatic diabetes; Clinical phenotype; Genetic risk factors

Core tip: Tropical calcific pancreatitis (TCP) is a form of chronic pancreatitis of unknown etiology. The phenotype of TCP is quite heterogeneous and has undergone a marked change over the last few decades, such that only a small fraction of such cases represent classical TCP. Several studies have shown the important role of genetic factors in the pathophysiology of TCP and provide evidence of genetic and mutational heterogeneity, compared with that in Western countries. Hence, it is important to understand the genotype-phenotype correlation in TCP. This review summarizes recent developments that provide some understanding of genotypic and phenotypic heterogeneity in this enigmatic disease.

INTRODUCTION

Pancreas is a heterocrine gland that is comprised of an

exocrine portion constituted by ductal cells and pancreatic acinar cells whose secretion is responsible for digestion of food, whereas the endocrine component is mainly involved in the maintenance of glucose homeostasis[1]. Pancreatic acinar cells synthesize, store and secrete the enzymes required for digestion of nutrients. Trypsin(ogen) is the key enzyme as it converts many proteolytic proenzymes into their active forms in the duodenum. Human pancreatic juice contains three isoforms of trypsinogen. On the basis of relative electrophoretic mobility, they are commonly referred to as cationic trypsinogen (*PRSS1*), anionic trypsinogen (*PRSS2*), and mesotrypsinogen (*PRSS3*). Cationic trypsinogen represents approximately two-thirds of total trypsinogen, while anionic trypsinogen makes up about one-third. Mesotrypsinogen is a minor form, accounting for less than 1% of trypsinogens or 0.5% of pancreatic juice proteins. It is thought that about 5% of trypsinogen is activated within the normal pancreas. However, there are several molecular and cellular mechanisms that have evolved to protect the pancreas from enzymes that are activated in the cell, especially proteases which could otherwise lead to auto-digestion of the pancreas leading to pancreatitis. These include compartmentalization of digestive enzymes in vacuoles like zymogen granules and lysosomes, the presence of pancreatic secretory trypsin inhibitor (PSTI)/serine protease inhibitor kazal type I (*SPINK1*) to antagonize the intrapancreatically activated trypsinogen, and maintenance of suboptimal intracellular pH and Ca^{2+} levels. In 1896, Chiari[1] proposed that intracellular activation of digestive enzymes, especially trypsinogens within the pancreas leads to pancreatitis[2].

Chronic pancreatitis (CP, OMIM 167800) is a progressive inflammatory disease of the pancreas that leads to persistent and irreversible morphological changes such as parenchymal fibrosis and calcification, cysts, necrosis and development of pancreatic stones. Continuous tissue destruction and remodeling often results in loss of exocrine and/or endocrine function. This manifests as two major clinical symptoms: upper abdominal pain and maldigestion. The prevalence of CP varies worldwide from 5-12/100000 individuals per year in United States to as high as 114-200/100000 individuals per year in southern India[3,4]. The TIGAR-O classification system first proposed in 2001 identifies 6 major etiologies for CP[5]. These include: toxic-metabolic factors, such as alcoholism, hypercalcemia, hyperlipidemia; idiopathic causes; genetic predisposition; an autoimmune response; recurrent and severe acute pancreatitis; and obstructive CP associated with pancreas divisum, sphincter of Oddi disorders, and others. Alcohol is the most common risk factor for CP reported in the West and some parts of Asia[4,6,7]. Certain proportions of CP patients do not possess any of the identified risk factors and thus are classified as having idiopathic CP (ICP). In addition, mutations in various candidate genes such as *SPINK1*, cationic and anionic trypsinogens (*PRSS1* and *PRSS2*), cystic fibrosis transmembrane regulator (*CFTR*), chymotrypsin C (*CTRC*)

and carboxypeptidase A1 (*CPA1*) have been reported with some variability in different parts of the world[8,9]. Historically, the form of CP that is prevalent in India is known as tropical calcific pancreatitis (TCP, OMIM 608189), described as a disease with "pain in childhood, diabetes in puberty, and death at the prime of life"[10]. Due to wide heterogeneity in symptoms at presentation, a large number of terminologies such as chronic calcific pancreatitis, fibro-calcific pancreatitis, chronic calcified pancreatitis, fibrocalculous pancreatitis and tropical pancreatitis have been used to describe this enigmatic entity. This has made us wonder whether it is the same disease with different names or different diseases with the same name. In this review, we summarize the recent developments and current status of the disease, make an attempt to explain the peculiarities and similarities with CP of other etiologies, and advance hypotheses to explain this dichotomy and its implications.

PHENOTYPE IN TROPICAL CALCIFIC PANCREATITIS

Although Zuidema first described TCP in young diabetics in Indonesia with fibrosis and calcification of the pancreas during the 1950s[11], it was the report by Geevarghese from Kerala in South West India describing young, malnourished patients with a cyanotic hue of the lips, bilaterally enlarged parotid gland, pot belly, and sometimes pedal edema, that caught the attention of the scientific community[10]. The clinical phenotype of TCP seems to have changed considerably since its first description. Recent reports have questioned the existence of classical TCP and instead used the terminology of ICP or simply CP[12]. Although there are clear-cut WHO-defined diagnostic criteria for TCP and fibrocalculous pancreatic diabetes (FCPD), the nomenclature is quite old[13]. Recent studies have defined the disease based on distinctive yet arbitrary features such as; onset at less than 30 years of age, a body mass index (BMI) less than 18 kg/m^2, absence of any other cause of pancreatitis, and presence of diabetes[12,14]. Based on these criteria, only 4%-6% of patients could be classified as having TCP in the study by Balakrishnan *et al*[14]. However, as indicated earlier[15], many of these points are debatable. Firstly, the use of BMI as a diagnostic criterion for classification of CP is contentious as a BMI value < 18.5 kg/m^2 suggests underweight status which may not be the same as malnourished. In addition, malnutrition has been shown to be an effect rather than a cause of CP. Furthermore, the abovementioned studies have sub-grouped ICP into early-onset ($\leq$ 30 years) and late-onset (> 30 years) and there are divided opinions over the proposal that those belonging to the early-onset category might resemble TCP[16,17].

In the following sections, we highlight the changes in the clinical profile of TCP observed over the last few decades. Several studies have also compared the features of TCP with alcoholic CP (ACP) that is more common

in Western countries.

Age of onset
The phenotype of TCP was initially described in children and in adolescents[10]. However, subsequent studies have reported a late age of onset of the disease. For instance, Balakrishnan *et al*[18] compared clinical characteristics of 220 TCP patients studied in the 1980s with another recent cohort of 244 patients, and found that both age at onset and age at presentation was nearly a decade later in the recent study than in the previous cohort. Similar observations have been made in other studies irrespective of the geographical location (North/South India)[14,16,17]. In addition, comparative studies of TCP/ICP patients with ACP patients reported a significantly earlier age of onset in the former[14,16,18].

Pain
Irrespective of the disease etiology, abdominal pain is the most predominant presenting complaint in the majority of CP patients. The characteristic pain is moderate-to-severe in intensity, begins in the epigastrium, and often radiates to the back. It may be relieved by sitting forward or leaning forward, by assuming the knee-chest position on one side, or by squatting and clasping the knees to the chest. Pain may increase after a meal and is often nocturnal. Quite frequently, it is associated with nausea and vomiting and occasionally with jaundice.

Macroscopic features of the pancreas
There seems to be heterogeneity in studies reporting the proportion of CP patients developing calcification. While studies from southern India observed calcification in more than 90% of patients[17,19], reports from northern India suggest it to range from 40%-80%[12,16,20]. Imaging studies using endoscopic retrograde cholagiopancreatography (ERCP) and ultrasonography have shown striking differences in the radiological appearance of TCP and ACP. While TCP is characterized by the presence of large, discrete, dense calculi that are usually intraductal, patients with ACP have typically small speckled parenchymal calculi with irregular, indefinite margins. The first ERCP study by Balakrishnan *et al*[21] in TCP reported that calcific TCP displays a greater degree of ductal derangement compared with the non-calcific variety. The study also noted that the changes in TCP were far more pronounced than those described in ACP. Another study observed that the degree of ductal dilation, pancreatic calculi, and pancreatic atrophy is higher in TCP compared with other forms of pancreatitis[22]. Similar observations have been made in a recent prospective nationwide study where calculi (60%-70%), a dilated pancreatic duct (55%-65%), and atrophy of the gland (30%-40%) were major imaging abnormalities[14].

Diabetes
Development of diabetes is known to be one of the common end points of TCP. Earlier, various terminolo-

gies such as pancreatic diabetes, pancreatogenous diabetes and tropical pancreatic diabetes were proposed for this form of diabetes. In order to maintain uniformity, the WHO Study Group proposed the use of the term FCPD in reference to diabetes secondary to disease of the exocrine pancreas[23]. One of the characteristic clinical features of FCPD is that, despite the requirement for insulin to control diabetes, the patients rarely become ketotic on withdrawal of insulin. This is attributed mainly to partial preservation of beta cell function as indicated by C-peptide studies[24,25]. Histopathology and immunohistochemistry of the pancreas in FCPD subjects shows atrophy of the pancreatic exocrine tissue and a decrease in the number of islets in some cases and hyperplasia in others[26]. Nesidioblastosis is also seen in some patients. Overall, there is a decrease in insulin positivity in islets that directly correlates with serum C-peptide levels and inversely with the duration of diabetes[26]. The series of patients reported by Geevarghese were actually cases of FCPD[10]. These patients were young (majority < 40 years of age at onset), poor, and extremely emaciated, which emphasized the presence of protein energy malnutrition. However, a recent study reported a decline in the proportion of FCPD cases, from 1.6% of all diabetic patients registered annually during the early 1990s to 0.2% during the period 2006-2010, while the prevalence of diabetes secondary to ACP has remained the same[27]. Additionally, there has been a significant increase in the BMI of FCPD subjects from 19.4 ± 3.6 kg/m^2 during 1991-1995 to 21.2 ± 3.8 kg/m^2 during 2006-2010 ($P < 0.001$)[27]. Also, a progressive increase in the age at diagnosis of FCPD patients has been observed during this period of study, while there was a decrease in age at diagnosis for diabetes secondary to ACP[27]. The prevalence of microvascular complications such as retinopathy, neuropathy, nephropathy, or microalbuminuria observed in FCPD is similar to that in type 2 diabetes[28].

Pancreatic cancer
The lifetime risk of developing pancreatic cancer in CP patients is reported to be around 4%[29]. Several retrospective and prospective studies to date report an increased association between TCP and occurrence of pancreatic cancer[30,31]. In the study by Ramesh *et al*[31], 22 out of 266 TCP patients (8.3%) presenting over an 8-year period had pancreatic adenocarcinoma. Factors associated with high risk for cancer were age > 40 years, short duration of symptoms, weight loss, presence of a mass on ultrasound, and ductal block on ERCP. These patients showed distinct features, such as younger mean age of onset, presence of calculi, and a higher incidence of diabetes compared to those with *de novo* pancreatic cancer. Chari *et al*[30] reported an increased risk of pancreatic cancer in TCP patients compared with the background pancreatic cancer rate (RR = 100.0, 95%CI: 37.0-218.0). A recent study also reported the frequency of malignancy in TCP patients to be around 4%[14]. The risk is thought to be generally higher in TCP compared with ACP.

ETIOPATHOGENESIS OF TCP

Since the first report of TCP[10], several factors have been proposed to be involved in its pathogenesis. The major hypotheses have revolved around malnutrition, cassava/cyanogen toxicity, oxidative stress and trace element deficiency, and familial and genetic factors.

Malnutrition

In earlier reports, TCP was primarily observed in poor, malnourished individuals, which led to the proposition that malnutrition might be an important causal factor for TCP[32]. However, recent observations have questioned this hypothesis. Recent prospective observational and case-control studies have reported that only 20%-25% TCP patients were undernourished before onset, and the majority of patients lost weight only after disease onset[33,34]. It has been argued that malnutrition thus could be the effect rather than the cause since TCP and consequent malabsorption could itself lead to severe weight loss[34]. Although data are convincing that malnutrition is not causally related to the etiopathogenesis of TCP, it is possible that malnutrition modulates the phenotype of the disease. High carbohydrate and low protein diets have also been shown to result in ductal changes, with mucoid metaplasia and parenchymal atrophy in an animal model in the bonnet monkey[35]. However, pancreatic changes were rather different from those typically seen in TCP, and the animals predominantly developed vascular and cardiac changes-features not observed in TCP patients[35].

Cassava/cyanogen toxicity

In several parts of the world, cassava (tapioca, *Manihot esculenta*) is consumed as a staple food by poor people. It is known to contain cyanogenic glycosides such as linamarin and lotaustralin, whose detoxification in the body requires sulfur. Malnourished individuals are deficient in sulfur-containing amino acids such as methionine and cysteine. Since cassava was a staple diet in Kerala, it gained the status of a co-trigger as a logical extension of the nutritional hypothesis. The cassava hypothesis has been discarded because: (1) cassava consumption was not found to be a risk factor in case-control studies including one from Kerala[36]; (2) patients with TCP have been reported from areas where cassava is not consumed[4,14]; and (3) long-term cassava consumption did not produce diabetes or pancreatitis in a rat model[37].

Micronutrient deficiency and oxidative stress

Multiple micronutrient deficiency is common in CP and likely to be related to its pathogenesis through its influence on oxidative stress. A study from Kerala has shown enhanced lipid peroxidation and decreased antioxidant status both in TCP and ACP[38]. The authors have further extended their earlier observation that zinc deficiency may also have a significant role to play in CP[39]. Zinc deficiency may occur as a result of pancreatic exocrine insufficiency. Moreover, zincuria has also been observed in most cases with pancreatic insufficiency[39,40].

Alcohol, smoking and other environmental toxins

Although data are variable, an increasing trend has been observed in the occurrence of ACP cases in India. Reports from southern India indicate a rise in cases of ACP from 2% during the 1980s to 33% over the last decade[17,18], whereas studies from northern India report a near equal (30%-40%) prevalence of ACP or predominance of TCP/ICP[4,12,16]. Even in the cases of TCP/ICP, a large proportion of individuals are alcohol drinkers. A majority of alcohol drinkers have also been reported to be smokers which further increases the risk of TCP[17,18]. Additionally, xenobiotic stress has also been implicated in the etiopathogenesis of TCP[41].

Familial aggregation and genetic factors

In one of the earliest studies, in 98 family members comprising 24 parents, 57 siblings and 17 offspring of TCP probands, familial aggregation was seen in 8% of TCP patients[42]. In some families, there was evidence of vertical transmission of TCP from parents to the offspring, while in others, horizontal distribution of the disease was observed. This suggests, but does not necessarily prove, a hereditary etiology for TCP since several family members could be exposed to the same toxic and/or other environmental factors. This led to the speculation that genes could be involved in the pathogenesis of TCP. However, it was only after the identification of cationic trypsinogen (*PRSS1,* OMIM 276000) as risk factor in Western populations that researchers focused on the role of genetic variants in TCP[43,44].

GENETIC HETEROGENEITY IN TCP

Trypsinogen(s)

As early as 1896, it was hypothesized that pancreatitis results from premature trypsinogen activation within the pancreas[2]. In 1996, independent familial linkage analysis studies mapped the gene locus on chromosome 7q35 and demonstrated its association with hereditary pancreatitis (HP)[45,46]. Subsequent fine mapping studies identified the c.365G>A (p.R122H) mutation in *PRSS1* to be associated with HP[43]. Since then, a large number of variants in *PRSS1* have been identified in CP patients[47]. Of these, p.R122H, p.N29I and p.A16V are most commonly reported, and their causality in CP through diverse mechanisms has been proven beyond doubt[48]. None of the mutations in *PRSS1* that have been reported to be associated with HP, and CP in Western populations has been found in TCP patients[49,50]. Mutations in anionic trypsinogen (*PRSS2*) were hypothesized to cause the disease by a mechanism similar to that of *PRSS1*. Earlier studies by various groups did not find association of any polymorphism of *PRSS2* with ICP and TCP patients[51-53]. The protective role of the p.G191R *PRSS2* mutation, identified in Europeans, has also been not replicated in Indians[54]. In addition, no copy number variation muta-

tion in *PRSS1/PRSS2* was found in TCP patients, suggesting that trypsinogen gene mutations do not play an important role in the pathogenesis of TCP in the Indian population[55].

Serine protease inhibitor Kazal type 1

PSTI encoded by *SPINK1* (OMIM 167790) is also synthesized in acinar cells of the exocrine pancreas. Because of its ability to trap up to 20% of the potential trypsin activity, SPINK1 has long been thought to constitute one of the defense mechanisms against prematurely activated trypsin within the pancreas. Identification of *SPINK1* as a susceptibility gene for CP[44] was followed by several reports confirming its association worldwide with various forms of pancreatitis. To date, more than 40 variants in *SPINK1* have been identified. The most commonly associated variant c.101G>A (p.N34S) has shown a strong association with TCP as well[49,50]. Similar associations of varying strength have been reported by several studies establishing *SPINK1* as a strong candidate for contributing to the pathogenesis of TCP[56-59]. Overall, these studies assessed 351 TCP patients and 973 controls. The high-risk haplotype around p.N34S was detected in 168 of 702 patient alleles and in 44 of 1946 control alleles. The pooled OR calculated using the random-effect model was 19.15 (95%CI: 8.83-41.56)[60]. However, no genotype-phenotype correlation has been found in patients carrying the p.N34S *SPINK1* mutation in homozygous or heterozygous state, and a wide variability has been reported in the pattern of inheritance. Hence, in contrast to the causal nature of *PRSS1* mutations, *SPINK1* has been accorded the role of disease modifier. A more recent study assessed the role of *SPINK1* promoter variants in the pathogenesis of CP[61]. A rare loss-of-function variant c.-142T>C that leads to disruption of the HNF1 binding site and hence reduced *SPINK1* expression was identified exclusively in TCP patients[61]. Another rare variant, c.-215G>T, also identified only in FCPD patients did not affect *SPINK1* expression. These results suggest that p.N34S *SPINK1* continues to be the strongest risk predictor for TCP.

Cathepsin B

Human cathepsin B (*CTSB*, OMIM 116810) is a 339 amino acid long thiol protease belonging to the peptidase C1 family. It is primarily localized to lysosomes and is involved in intracellular degradation and turnover of proteins. Nearly 30 years ago, it was speculated that lysosomal enzymes might play a role in the pathophysiology of pancreatitis[62]. This was because an earlier study had shown that at least one lysosomal hydrolase, cathepsin B, was capable of activating trypsinogen[63], which has been supported by several subsequent studies[64,65]. Additionally, it has been shown that supramaximal stimulation causes redistribution of lysosomal enzymes leading to their co-localization with digestive enzyme zymogens within intra-acinar cytoplasmic vacuoles[66]. These observations made *CTSB* an interesting candidate gene. Indeed, a study involving 306 TCP patients and 330 controls reported

that polymorphism p.L26V *CTSB* was associated with TCP (OR = 2.09, 95%CI: 1.55-2.81; *P* = 0.013)[67]. In addition to the p.L26V variant, the polymorphism p.S53G also had a significantly different distribution in p.N34S SPINK1 carriers and non-carriers. These variants, which lie in the pro-peptide region of CTSB, were proposed to lead to mis-localization of cathepsin B to zymogen granules, thus causing premature activation of trypsinogen. However, a recent study conducted in a moderate sample size of 150 cases and 150 controls from North India failed to replicate this association[68]. Surprisingly, the mutant allele frequency of 0.33% in controls reported in the study is far lower than that of 30% reported earlier[67], which raises doubts on the veracity of the results

Chymotrypsin C

The human chymotrypsin C gene (*CTRC*; OMIM 601405) encodes a 268 amino acid long serine protease (a member of the peptidase S1 family) that is secreted from the pancreas and has a chymotrypsin-like protease activity. Recent studies have demonstrated that auto-activation of trypsinogens in humans is proteolytically regulated by CTRC through two independent and seemingly conflicting mechanisms. On one hand, CTRC stimulates the autoactivation of cationic trypsinogen by cleavage at the Phe18-Asp19 peptide bond[69], while on the other hand, it promotes degradation of all human trypsin and trypsinogen isoforms with high specificity by cleaving the Leu81-Glu82 in the calcium binding loop[70]. The act of activation and degradation of trypsinogen by CTRC is regulated by prevailing Ca^{2+} concentrations[70]. Since the intra-acinar activation of cationic trypsinogen is thought to be the primary cause of pancreatitis, impairment of the CTRC-dependent regulation of auto-activation of trypsinogen increases the risk of intra-pancreatic trypsinogen activation and consequent pancreatitis in humans. Two initial studies investigated the role of *CTRC* variants in patients of Indian origin and found them to be associated with TCP[71,72]. However, both studies focused on a specific region of the gene in a small number of subjects. Subsequently, a comprehensive study that screened the whole *CTRC* gene in a large, ethnically matched case-control CP cohort, including TCP, observed significant over-representation of rare *CTRC* variants in CP patients compared with normal individuals[73]. Non-synonymous variants, c.217G>A (p.A73T) and c.703G>A (p.V235I), were the major risk predictors, in comparison to the c.738_761del24 (p.K247_R254del) and c.760C>T (p.R254W) variants that were the predominant mutations in European CP patients. While p.A73T exhibits its pathogenicity by eliciting endoplasmic reticulum stress[74], p.V235I is known to reduce activity and secretion of the protein[75]. In addition, a synonymous variant, c.180C>T [p.(=)], was also found to be significantly associated with CP (OR = 9.89, 95%CI: 2.95-33.18; *P* = 5.9 × 10⁻⁶). Interestingly, the spectrum of *CTRC* mutations identified in TCP patients was similar in all three studies, but entirely different from that observed in Western CP patients.

Based upon the biochemical activities of CTRC and the functional properties of *CTRC* variants, three mutually non-exclusive models for explaining the role of *CTRC* variants in predisposing to CP were put forward[76]: (1) impaired trypsinogen and/or trypsin degradation; (2) induction of endoplasmic reticulum stress; and (3) impaired activation of A-type carboxypeptidases.

Carboxypeptidase A1

The last hypothesis as mentioned above is of special interest because, based on functional evidence, a recent study proposed that CTRC is a physiological co-activator of pro-carboxypeptidase A1 (proCPA1) and pro-carboxypeptidase A2 (proCPA2)[77]. After trypsinogens, proCPA1 is the second largest component of pancreatic juice, contributing more than 10% of the total protein. Indeed, genetic and functional data from a recent study has established the global role of *CPA1* variants in the pathogenesis of CP, including TCP[9]. However, there was evidence of heterogeneity in the spectrum of mutations identified in different populations. In the individuals of Indian origin, three non-synonymous variants (p.D32H, p.R169H, and p.Y308H) were novel and present exclusively in patients, while the frequency of p.A208T was comparable between cases and controls. Apparent activities of p.D32H, p.R169H and p.Y308H were 79%, 24%, and 3%, respectively, of the wild protein, whereas their respective relative secretion levels were 75%, 23%, and 17%, respectively, of the native protein. This confirms the earlier notion that the mutational spectrum in various CP-associated genes is different in TCP than in other types of CP in the Western world.

Cystic fibrosis transmembrane regulator

The *CFTR* gene encodes a member of the ATP-binding cassette transporter superfamily. In the pancreatic duct, CFTR couples functionally to the anion exchangers to generate bicarbonate secretion for alkalinizing the duodenal lumen[78]. Abnormal *CFTR* genotypes are strongly associated with cystic fibrosis (CF)[79]. Considering the fact that patients with CF occasionally suffer from pancreatitis; pancreatic pathology in CP and cystic fibrosis shows intraductal plugging; also, CP is a known cause of false positive sweat tests, and two studies in 1998 simultaneously reported an association between *CFTR* mutations and CP[80,81]. Only two studies have investigated the role of *CFTR* variants in TCP. In the study by Bhatia and colleagues, all 27 *CFTR* coding exons were analyzed in 18 Indian TCP patients[82]. Two patients (11%) showed a *CFTR* variant: one subject was homozygous for the 5T allele and the other heterozygous for p.R1070Q, which is presumed to be a mild missense variant. The overall frequency of *CFTR* alterations was 0.083 (3/36), which was far lower than that observed in white Caucasian subjects with CP (range: 0.20-0.24). In a more recent study, mutations in 19 of 27 exons of the *CFTR* were analyzed in 100 TCP patients and 100 healthy controls[12]. A total of 21 severe and mild *CFTR* variants (including six novel variants) were detected in 50% of patients compared with two different variants in 10% of controls ($P < 0.0001$). Of these, 27 patients were trans-heterozygous for *CFTR* variants and the p.N34S *SPINK1* mutation[12].

Pancreatic stone protein

An important feature of TCP is the high incidence of pancreatic calcification and stone formation. Human Reg protein is encoded by the pancreatic stone protein (*reg1a*) gene (regenerating gene) as a 166 amino acid pre-proprotein with a 22-residue long signal sequence, and is highly represented in the human pancreatic secretions. It was first isolated as a major protein component of pancreatic stones in patients with ACP and hence called pancreatic stone protein. It was suggested that it could promote the nucleation of calcite crystals or prevent pancreatic lithiasis by inhibiting calcite crystal nucleation and growth in the pancreatic juice[83]. With suggestions that it might help in preventing the harmful activation of protease precursors in the pancreatic juice, it was speculated that mutations in this gene could lead to pancreatitis and calcification. However, no association with TCP could be established even on screening of all exons of the *Reg1a* gene (OMIM 167770)[84,85]. As the protein is known to be downregulated in TCP patients, a recent study screened the gene, including the putative promoter and intronic regions, but did not find a significant association with TCP[86].

Glycoprotein 2

The glycoprotein 2 (*GP2*) gene is specifically expressed in the pancreatic acinar cells and represents the major component (about 40%) of the total zymogen granule (ZG) membrane protein[87]. During the secretory process, GP2 is cleaved from the membrane by phosphatidylinositol-specific phospholipase C and is secreted into the duct lumen along with other digestive zymogens. In addition, a soluble form of GP2 is also present in the content of ZGs. Given the fact that intra-ductal plug formation is one of the early events in the development of CP and GP2 is found to be a major component of these plugs[88], it was hypothesized that variations in *GP2* may potentially affect the risk of duct obstruction and CP. Mutational screening of exons 3 and 9 of the *GP2* gene in TCP patients identified two variants of which the variant c.1275A>G showed a disease predisposing effect[89]. A recent study has demonstrated that in the presence of this variant, the ratio of full-length transcript:total transcript is much lower than that derived from the wild type[90]. This is because the c.1275A>G variant significantly reduces the rate of exon 9 inclusion compared with the wild-type sequence[90]. It results in substitution of the last 116 amino acids by 15 new amino acids. These changes may lead to structural alterations and hence compromise the function of the protein[90].

Transcription factor 7-like 2

Progression of TCP to diabetes, also known as FCPD, occurs in a majority of TCP patients. However, the nature of diabetes associated with pancreatitis is contro-

versial since it shows features of both type 1 (T1D) and type 2 (T2D) diabetes. A recent study from our group hypothesized that the type and mechanism of diabetes in FCPD patients can be understood by investigating a known genetic susceptibility factor for T1D or T2D[91]. In this study, T2D-associated polymorphisms in transcription factor 7-like protein 2 (TCF7L2, OMIM 602228) were screened in TCP and FCPD patients. Although no independent association with FCPD was identified, data suggested that polymorphisms in *TCF7L2* might interact with *SPINK1* and *CTSB* mutations and cause FCPD[91].

Calcium sensing receptor

Experimental evidence suggests that intracellular and extracellular calcium levels play an important role in the initiation of protease activation within the pancreas. The function of calcium sensing receptor (CASR) is to sense small differences in the circulating calcium levels. Mutations involving *CASR* (OMIM 601199) have been proposed to increase the risk of CP, since high intracellular levels of calcium activate trypsinogen within the acinar cells. A combination of *CASR* and *SPINK1* gene mutations has also been proposed to predispose to ICP. Another study conducted in India pertaining to TCP patients identified four novel mutations (p.P163R, p.I427S, p.D433H, p.V477A) in *CASR*[92]. A combination of both the p.N34S *SPINK1* mutation and *CASR* mutations was seen in approximately 6% (2/35) of the patients, while 22% (6/35) harbored a single mutation[92]. However, the drawback of this study was that a limited number of patients and controls were screened.

CONCLUSION

The clinical phenotype of TCP has changed over the years. The disease which was common in young adults and adolescents is now reported to occur in relatively older people with both the age at onset and age at presentation being nearly a decade later now than in the previous studies. In addition, the presentation of the disease has become more heterogeneous. Only a fraction of cases now satisfy the criteria of classical TCP[12,14]. This change has been attributed to socioeconomic, dietary, and lifestyle changes over the past 20-30 years. Instead, there have been rising trends in alcohol consumption and smoking habits in young Indians. As a result ACP now comprises nearly one-third of CP patients in India[17,18]. It has been reported that classical TCP in India now presents as ICP whose phenotype is somewhat similar to that reported from other countries. The results from candidate gene studies established that several genetic components are involved in the pathophysiology of TCP, and there is evidence of genetic and mutational heterogeneity between TCP and CP in Western populations. These components work both *via* trypsin-dependent as well as trypsin-independent pathways. Overall, these observations point to the fact that TCP is indeed a complex multifactorial disease and in-depth studies are needed to dissect the role of individual factors and their interaction in the pathophysiology of the disease.

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Oxidative stress and inflammatory signaling in cerulein pancreatitis

Ji Hoon Yu, Hyeyoung Kim

Ji Hoon Yu, New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 701-310, South Korea
Hyeyoung Kim, Department of Food and Nutrition, Brain Korea 21 PLUS Project, College of Human Ecology, Yonsei University, Seoul 120-749, South Korea
Author contributions: All authors contributed to the manuscript.

Correspondence to: Hyeyoung Kim, PhD, Department of Food and Nutrition, Brain Korea 21 PLUS Project, College of Human Ecology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-749, South Korea. kim626@yonsei.ac.kr
Telephone: +82-2-21233125 Fax: +82-2-3645781

Abstract

Oxidative stress is considered to be an important regulator of the pathogenesis of acute pancreatitis. Reactive oxygen species (ROS) regulate the activation of inflammatory cascades, the recruitment of inflammatory cells and tissue damage in acute pancreatitis. A hallmark of the inflammatory response in pancreatitis is the induction of cytokine expression, which is regulated by a number of signaling molecules including oxidant-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), signal transducer and activator of transcription 3 (STAT3), and mitogen-activated protein kinases (MAPKs). Cross-talk between ROS and pro-inflammatory cytokines is mediated by NF-κB, AP-1, STAT3, and MAPKs; this crosstalk amplifies the inflammatory cascade in acute pancreatitis. Therapeutic studies have shown that antioxidants and natural compounds can have beneficial effects for patients with pancreatitis and can also influence the expression of proinflammatory cytokines in cerulein-induced pancreatitis. Since oxidative stress may activate inflammatory signaling pathways and contribute to the development of pancreatitis, antioxidant therapy may alleviate the symptoms or prevent the development of pancreatitis. Since chronic administration of high doses of antioxidants may have deleterious effects, dosage levels and duration of antioxidant treatment should be carefully determined.

Key words: Reactive oxygen species; Inflammatory signaling; Cerulein pancreatitis

Core tip: The pathogenesis of acute pancreatitis is not completely elucidated. Oxidative stress may contribute to the development of acute pancreatitis. Evidence supporting the role of reactive oxygen species and cytokines as a risk for pancreatitis and the concept of antioxidant supplementation as a preventive approach for pancreatitis has been proposed. Here we review the literature on oxidative stress, cytokine expression, inflammatory signaling, and natural antioxidant supplementation using an experimental model of cerulein-induced acute pancreatitis.

INTRODUCTION

Acute pancreatitis is a disease characterized by the activation of digestive proteases, inflammatory infiltration of macrophages and neutrophils, and necrosis of the pancreatic tissue. High doses of a cholecystokinin (CCK) analogue, cerulein, have been shown to stimulate the

maximum secretion of pancreatic amylase and lipase[1]. This increased secretion results in pancreatitis, which is characterized by cytoplasmic vacuolization, the death of acinar cells, edema formation, and infiltration of inflammatory cells into the pancreas[2]. Interestingly, neutrophils obtained from patients with acute pancreatitis have been shown to exert enhanced production of reactive oxygen species (ROS)[3]. ROS have been proposed to play a critical role in the pathogenesis and development of acute pancreatitis. The major source of ROS in acute inflammation appears to be the NADPH oxidases; on the other hand, the major target of ROS and redox signaling in acute pancreatitis is nuclear factor-κB (NF-κB)[4,5]. Both the activation of NF-κB and the NF-κB-regulated expression of interleukin-1β (IL-1β), IL-6, and TNF-α have been shown to be involved in initiation and aggravation of acute pancreatitis. Studies focusing on natural compounds, have shown that caffeine-free extract from green tea reduces the degree of acute pancreatitis, the activation of NF-κB, and reduces the expression of pro-inflammatory cytokines[6]. Other antioxidants such as ascorbic acid and N-acetyl cysteine (NAC) have also been shown to exert beneficial effects against acinar cell degeneration, pancreatic edema, intracellular vacuolization and inflammatory infiltration in cerulein-induced pancreatitis[7]. This review will focus on the involvement of ROS in inflammatory signaling pathways in the context of the cerulein-induced acute pancreatitis model. In addition, natural compounds that may alleviate the symptoms or prevent the development of pancreatitis will also be discussed.

ROS SIGNALING IN CERULEIN-INDUCED ACUTE PANCREATITIS

Depletion of pancreatic glutathione (GSH) has been shown to be involved in the early phase of acute pancreatitis[8] and also to influence the extent of disease severity[9]. The activities of multiple antioxidant enzymes, including glutathione peroxidase, superoxide dismutase (SOD), and catalase, decrease in the course of pancreatitis; the levels of antioxidant vitamins have also been shown to decrease[10,11]. Moreover, the level of pancreatic glutathione peroxidase is reduced both in cerulein-induced acute pancreatitis models[7] and in patients with acute pancreatitis[12]. The serum level of thioredoxin-1, an antioxidant, has been shown to increase in patients with severe acute pancreatitis[13]. However, overexpression of thioredoxin-1 has been shown to attenuate the inflammatory response in acute pancreatitis[14]. Interestingly, cerulein-induced pancreatitis induces expression of metallothionein-1, and overexpression of metallothionein-1 has been shown to protect against pancreatic damage after induction of pancreatitis in mice[15]. Thus, oxidative stress appears to regulate the early phase of acute pancreatitis, since an improved antioxidant status is associated with improved clinical outcomes in patients with acute pancreatitis. The major source of ROS in inflammation has

been reported to be NADPH oxidases[4,5,16]. Deficient production of NADPH oxidase was shown to reduce trypsin activation in mice with cerulein-induced pancreatitis[17]; moreover, the NADPH oxidase NOX1 has been demonstrated to play a critical role in the induction of IL-6 expression and apoptosis in pancreatic AR42J acinar cells stimulated with cerulein[18].

INFLAMMATORY RESPONSE AND SIGNALING IN ACUTE PANCREATITIS

Hyper-stimulation of the CCK receptor, using supramaximal doses of the CCK analogue cerulein, has been shown to lead to NF-κB activation in pancreatic acinar cells[19]. Cerulein also produces ROS by activating the NADPH oxidase, NOX1, in pancreatic acinar cells[18]. Cerulein-mediated induction of acute pancreatitis is known to trigger NF-κB activation; this effect can be attenuated by pretreatment with NAC[19]. Pro-inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), play a major role in the inflammatory response associated with acute pancreatitis[20-23]. Antioxidants inhibit the expression of these inflammatory cytokines by suppressing NF-κB activation[21]. Clinical studies have revealed the presence of inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the sera of patients with acute pancreatitis. Patients with pancreatitis have been shown to have enhanced NF-κB activity; moreover, inhibiting NF-κB has been shown to reduce the inflammatory effects of pancreatitis[24]. Both experimental and clinical studies have implicated a role for NF-κB in the pathogenesis of acute pancreatitis[24,25]. Conditional overexpression of IκB kinase, a molecule which helps activate NF-κB by phosphorylating its inhibitory protein IκBα, has been shown to induce an inflammatory response in mice with acute pancreatitis[26]. Moreover, genetic silencing of NF-κB was shown to reduce the extent of pancreatic damage and to down-regulate the expression of TNF-α in cerulein-induced acute pancreatitis[27]. TNF-α and IL-1β are considered to be the primary cytokines in acute pancreatitis, since these cytokines initiate and propagate most of the consequences of the systemic inflammatory response[28]. These two cytokines also amplify the inflammatory cascade by activating mitogen-activated protein kinases (MAPKs) and NF-κB, which in turn induces the release of chemokines and other cytokines. The induction of chemokines occurs via a positive feedback loop, in which each chemokine also up-regulates its own expression[29]. Serum IL-6 levels have also been shown to be increased in patients with acute pancreatitis, these levels also correlate with disease severity[30]. In an IL-6 transgenic mouse model, cerulein-induced acute pancreatitis was shown to be more severe than in wild-type mice[31]. Cerulein-induced expression of IL-8 has also been reported to be regulated by NF-κB, AP-1, and MAPKs in pancreatic acinar cells[32]. A recent study showed that the janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway is activated by the CCK2 receptor in

pancreatic AR42J cells[33]. High doses of cerulein have also been shown to trigger phosphorylation of JAK2 and STAT3 in pancreatic acinar cells[34]. Moreover, inhibition of JAK2 and STAT3 via the anti-inflammatory properties of peroxisome proliferator activated receptor-γ (PPAR-γ) ligands, such as 15-deoxy-Delta-(12,14)-prostaglandin J2 (15dPG-J2) and troglitazone, has been shown to reduce the expression of IL-6[34]. Recent study shows that cerulein induces oxidative injury, inflammatory cytokines, and nucleosome release in pancreatic tissues and acinar cells of mice with pancreas-specific disruption in HMGB1 (high mobility group box 1)[35]. Treatment of NAC attenuates cerulein-induced pancreatic injury in these mice[35], suggesting that intracellular HMGB1 may prevent oxidative injury of pancreas and limit acute pancreatitis.

EFFECTS OF ANTIOXIDANTS AND NATURAL COMPOUNDS IN ACUTE PANCREATITIS

An antioxidant mixture was shown to reduce the level of malondialdehyde, and increase the activities of catalase and glutathione peroxidase in L-arginine-induced pancreatitis rats. Moreover, supplementation with an antioxidant mixture containing NAC, selenium, and vitamin C was shown to reduce pancreatic injury in rats[36]. Furthermore, treatment with NAC alone was sufficient to attenuate sodium taurocholate-induced pancreatitis in rats[37]. A combination treatment of ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one], which is a mimic of GSH peroxidase, and ethylhydroxyethyl cellulose (EHEC) was also shown to attenuate severe acute pancreatitis in rats[38]. Resveratrol, a plant-derived polyphenolic phytoalexin, has also be shown to reduce the expression of TNF-α and IL-8 by inhibiting NF-κB signaling in acute pancreatitis[39]. In the early stage of acute pancreatitis, NF-κB is activated in macrophages, which then produce cytokines. During acute pancreatitis, treatment with resveratrol reduces the expression of IL-1β and TNF-α in macrophages via NF-κB signaling pathways[40]. In cerulein-induced acute pancreatitis, treatment with resveratrol has been shown to prevent tissue damage, reduce the expression of IL-1β, and induce the expression of IL-10, an anti-inflammatory cytokine[41]. The effect of resveratrol may be due to its antioxidant effect with induction of catalase and MnSOD[42]. Moreover, cerulein-induced upregulation of IL-1β and TNF-α and depletion of GSH are rescued by treatment with lycopene, a natural carotenoid[43]. Glycyrrhizin treatment of acute pancreatitis has also been shown to suppress the production of proinflammatory cytokines (IL-6, IL-1β and TNF-α) and to stimulate recovery from histological changes such as acinar cell necrosis, hemorrhage, and edema[44]. Glycyrrhizin treatment has been shown to not only decrease the serum levels of MCP-1 and MIP-2 in cerulein-induced acute pancreatitis, but also to reduce the number of infiltrated granulocytes and monocytes in pancreatic tissues[45]. Glycyrrhizin exerted antioxidant effects and reduced activation of NF-κB, c-Jun N-terminal

kinase (JNK), and p38, redox-sensitive signaling events known to be relevant for influenza A virus replication[46]. Glycyrrhizin treatment decreased the incidence of free radical-induced lipid peroxidation and improved immunity activities in the blood and nasal mucosa of allergic rhinitis mice[47]. In addition, bioflavonoid curcumin, the pigment in turmeric (*Curcuma longa*), inhibited the activation of NF-κB and the expression of TNF-α and thus ameliorated cerulein pancreatitis in mice[48].

Antioxidant therapy is believed to have great potential, since its therapeutic efficacy has already been demonstrated in experimental acute pancreatitis. Patients admitted within 72 h of onset of pain were randomized to receive either placebo or antioxidants (vitamin C 500 mg, NAC 200 mg 8 hourly and antoxyl forte 1 capsule hourly with standard medical treatment) daily[49]. Treatment with vitamin C and NAC was shown to decrease oxidative stress and to improve the antioxidant status of 23 patients with acute pancreatitis[49]. Moreover, antioxidant therapy with selenium and D-α-tocopherol in 99 patients showed beneficial effects against necrotizing or mild acute pancreatitis[50]. A combination therapy of daily doses of antioxidants including 600 μg organic selenium, 9000 IU β-carotene, 0.54 g vitamin C, 270 IU vitamin E, and 2 g methionine was studied in three controlled clinical trials[51-53]. After treatment with a combination of antioxidants to 28 patients with idiopathic chronic, alcoholic chronic, or idiopathic acute pancreatitis, recurrent attacks and pancreatic pain were significantly attenuated[51]. Another study with 36 chronic pancreatitis patients, pain was reduced after the combination therapy and quality of life, physical, social functioning and health perception were enhanced[52]. In clinical trial with 147 patients, the antioxidants were administered for 6 mo, and pain and hospitalization were reduced[53]. Twenty patients with chronic pancreatitis received 500 mg curcumin with 5 mg of piperine or placebo for 6 wk[54]. Treatment of curcumin reduced erythrocyte malondialdehyde levels compared to placebo[54]. Bolus intravenous administration of vitamin C (10 g/d) for 5 d has been shown to alleviate pancreatitis symptoms, enhance the cure rate, reduce the complications, and decrease the length of hospital stays in 84 patients with acute pancreatitis[55].

In contrast, a multidrug approach was investigated in a randomized control trial using intravenous NAC, selenium and vitamin C in 43 patients with severe acute pancreatitis for 7 d. While makers for oxidative stress were lower in the treatment group, there was no significant difference in patient outcomes[56]. The study shows a lack of benefit from antioxidant therapy in severe acute pancreatitis. In another randomized study with multiple antioxidants, 39 patients with severe acute pancreatitis were randomized to standard treatment or standard treatment and vitamin C (1000 mg in 100 mL normal saline), vitamin E (200 mg oral), and vitamin A (10000 IU intramuscularly) for 14 d. No significant difference was demonstrated in the two treatment groups regarding, length of hospital stay and organ dysfunction[57]. Also, antioxidants therapy does not seem to confer protection in patients with
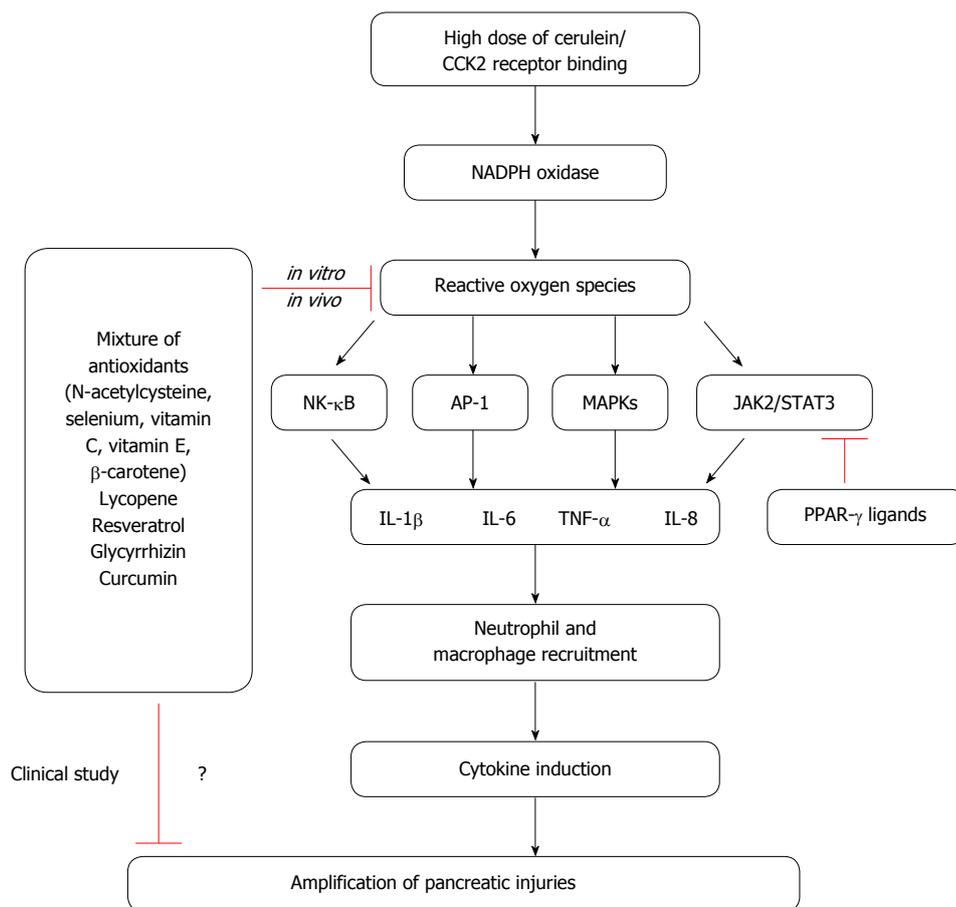
Figure 1 Scheme of oxidative stress-induced inflammation in cerulein pancreatitis. High dose of cerulein, a cholecystokinin (CCK) analogue, binds to CCK2 receptor and stimulates the activation of NAPH oxidase to produce reactive oxygen species. Reactive oxygen species activate redox-sensitive transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) as well as inflammatory mediators mitogen-activated protein kinases (MAPKs) (mitogen-activated protein kinases) and janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), which in turn induces the expression of cytokines IL-1β, IL-6, IL-8, and TNF-α in pancreas. Induction of cytokines recruits neutrophils and macrophages in the injured pancreatic tissues. Cytokines/chemokines act as a positive feedback loop to up-regulates their own expression. Therefore, cytokines positively regulate the induction of cytokines and pancreatic injuries are amplified. Peroxisome proliferator activated receptor-γ (PPAR-γ) ligands inhibit the activation of JAK2/STAT3 and suppresses inflammatory signaling in pancreas. Antioxidant nutrients and natural compounds reduce the levels of reactive oxygen species and suppress activation of NF-κB, AP-1, MAPKs, and JAK2/STAT3 to inhibit induction of cytokines and pancreatic injuries in experimental pancreatitis (*in vitro*, *in vivo*). Some clinical studies show a lack of benefit from antioxidant therapy while others have beneficial effects against acute and chronic pancreatitis by alleviating symptoms and enhancing the cure rate.

post endoscopic retrograde cholangiopancreatography (ERCP)-induced pancreatitis. In a double-blinded trial, patients were given a single dose (2 g) of β-carotene 12 hours prior to an ERCP. There was no difference in the incidence of acute pancreatitis between the patients who received antioxidant (9.4%) with those who had received placebo and developed (10%)[58]. Even though there has been no report for adverse effect directly attributable to antioxidant therapy, we could not exclude possible deleterious effects of chronic administration of high doses of antioxidants. Therefore, dosage levels and duration of antioxidant treatment should be carefully determined. The role of oxidative stress on inflammatory signaling and anti-inflammatory effects of natural compounds in cerulein-induced pancreatitis are summarized in Figure 1.

CONCLUSION

Oxidative stress is well established to increase throughout the course of pancreatitis. Furthermore, ROS are known

to mediate the activation of NF-κB, AP-1, MAPKs, and STAT3 in pancreatic acinar cells stimulated with cerulein. Crosstalk between ROS and pro-inflammatory cytokines, which is mediated by NF-κB, STAT3, and MAPKs, is believed to contribute to the inflammatory process in pancreas. Antioxidant combinations of NAC, organic selenium, β-carotene, vitamin C, and vitamin E may inhibit cytokine expression and thus reduce the severity of pancreatitis. Natural compounds with antioxidant effects, such as lycopene, resveratrol, and glycyrrhizin, also reduce the expression of inflammatory cytokines (TNF-α, IL-6, and IL-8) by suppressing NF-κB signaling in acute pancreatitis. Cumulatively, these studies demonstrate that oxidative stress plays an important role in the activation of inflammatory signaling pathways and in the pathogenesis of pancreatitis. Therefore, reducing the levels of ROS by antioxidant therapy may be clinically valuable for the treatment and/or prevention of pancreatitis. However, dosage levels and duration of antioxidant treatment should be carefully determined to prevent possible side

effects to the patients with acute and chronic pancreatitis.

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Fluid resuscitation in acute pancreatitis

Aakash Aggarwal, Manish Manrai, Rakesh Kochhar

Aakash Aggarwal, Department of Internal Medicine, SUNY Upstate, Syracuse, NY 13210, United States
Manish Manrai, Rakesh Kochhar, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India
Author contributions: Aggarwal A, Manrai M and Kochhar R wrote the paper; and Manrai M performed the literature review.
Correspondence to: Rakesh Kochhar, MD, Department of Gastroenterology, Postgraduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012,
India. dr_kochhar@hotmail.com
Telephone: +91-172-2756608 Fax: +91-172-2744401
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Abstract

Acute pancreatitis remains a clinical challenge, despite an exponential increase in our knowledge of its complex pathophysiological changes. Early fluid therapy is the cornerstone of treatment and is universally recommended; however, there is a lack of consensus regarding the type, rate, amount and end points of fluid replacement. Further confusion is added with the newer studies reporting better results with controlled fluid therapy. This review focuses on the pathophysiology of fluid depletion in acute pancreatitis, as well as the rationale for fluid replacement, the type, optimal amount, rate of infusion and monitoring of such patients. The basic goal of fluid epletion should be to prevent or minimize the systemic response to inflammatory markers. For this review, various studies and reviews were critically evaluated, along with authors' recommendations, for predicted severe or severe pancreatitis based on the available evidence.

Key words: Acute pancreatitis; Fluid resuscitation; Aggressive fluid therapy; Crystalloids; Colloids

Core tip: Acute pancreatitis can manifest as a severe form, which has a high mortality rate. The treatment of AP is primarily supportive, and fluid replacement therapy has emerged as one of the key treatment strategies. There is a lack of randomized studies addressing the questions of the best type of fluid, amount of fluid and rate of fluid transfusion. This paper reviews the available literature and the controversies and attempts to frame guidelines for fluid therapy in acute pancreatitis.

INTRODUCTION

Acute pancreatitis (AP) is acute inflammation of the pancreas, and has high morbidity and mortality rates[1]. AP displays a wide spectrum of disease presentation, ranging from self-limiting mild illness to rapidly progressive severe illness ending in multi-organ failure with a high risk of mortality. Different stages of severity have been described in the Atlanta classification[2]. It has been estimated that about 10% to 20% of AP patients develop the severe form, which has a 15% to 40% mortality rate[3].

A major factor complicating the appropriate management of AP is the failure to discriminate its mild and severe forms in the initial stages. This issue is critical, as about half of the patients with severe AP die within the first week due to the development of organ failure; the incidence of organ failure is maximal (17%) on the first day[4,5]. The causes for later mortality are development of infected necrosis and other complications. Thus, it is important to identify factors that can predict severity of the AP disease so as to guide early clinical management within the so-called interventional window[6,7].

Despite increased understanding of the underlying

pathophysiology of both the disease and its complications, its management remains a clinical challenge and is primarily based on supportive therapy[8]. Fluid resuscitation is the current cornerstone of early management, although there is little consensus on the details of its application[9]. Widely accepted clinical practice guidelines recommend vigorous fluid resuscitation in the early management of AP[10-12] (Table 1). However, there is a lack of consensus on specific recommendations regarding the type of fluid, optimal rate of fluid administration and end points to indicate adequate resuscitation[13].

Although aggressive fluid therapy is the cornerstone of treatment in AP, a few recent studies have suggested that non-aggressive fluid therapy may be better in reducing mortality and improving outcomes[14-18]. A recent systematic review has brought into focus the lack of evidence in literature on the type of fluid, rate of fluid infusion and the goals to be monitored[9]. Even the evidence that is available is of low or very low quality. Therefore, we reviewed the literature regarding fluid resuscitation in the course of AP, placing emphasis on goals, choice and amount of fluids to reduce complications such as pancreatic necrosis and organ failure (Table 2). The review's findings, written up herein, are based on a detailed PubMed search which encompassed all published articles up to the end of June 2014.

PATHOPHYSIOLOGICAL BASIS

The major etiologic factors of AP are alcohol and biliary obstruction; other causes being autoimmunity, drugs, trauma, iatrogenic and idiopathic. A major complication of the disease is organ failure as a result of excessive activation of a systemic inflammatory response cascade[19]. Pancreatic insult due to any etiology leads to release of pro-inflammatory mediators, such as zymogens, cytokines and vasoactive factors. These mediators cause endothelial cell activation leading to arteriolar vasoconstriction, increased permeability and circulatory stasis, thereby inducing ischemia[20]. This increased permeability, related to capillary leakage, causes intravascular fluid loss and hypotension, and shock may ensue (Figure 1). Further, accumulation of inflammatory mediators with enhanced leucocytes and endothelial interaction results in the activation of the coagulation cascade and hyper-coagulation. Micro-vascular thrombosis further causes tissue hypoxia and, ultimately, systemic inflammatory response syndrome (SIRS)[20]. Organ dysfunction usually occurs quite early in the course of severe AP, usually the first four days[21,22], and unless aggressive management is performed, it causes mortality in about 50% of cases within the first week of its manifestation[23]. The first five days after the onset of acute disease are considered as the "therapeutic interventional window"[6], during which aggressive fluid resuscitation can correct the third space losses and increase tissue perfusion. SIRS may be averted with prevention of multiple organ failure and/or

pancreatic necrosis.

CLASSIFICATION OF ACUTE PANCREATITIS SEVERITY

Severity of AP can be classified as mild (having no local or systemic complications), moderate (in which local or systemic complications are associated with organ failure that resolves within 48 h) or severe (in which organ failure persists beyond 48 h)[3]. The risk of mortality is nil or minimal in patients with no or minimal organ failure, and it can be as high as 15% to 40% in those with severe disease[11]. Organ failure has emerged as a key determinant of the severity of AP. Organ failure itself is classified as transient or persistent depending upon the duration and is classified as per the modified Marshall score[24].

There are various scoring criteria described in the literature to assess the severity of pancreatitis[25-30]. However, these scoring systems require 48 h to complete. The first 12-24 h are critical, as the maximum incidence of development of organ failure fits within this time frame[31]. Hence, two new scoring systems have been proposed recently, each of which assesses severity in the first 24 h. The Bedside Index of Severity in Acute Pancreatitis (BISAP) is one and assesses 5 criteria: blood urea nitrogen (BUN) > 25 mg/dL, age > 60 years, impaired mental status, SIRS and pleural effusion[32]. For BISAP, a score of > 2 is associated with a 10-fold increase in mortality risk[27,33]. The second scoring system is the Harmless Acute Pancreatitis Score, which focuses on patients who are unlikely to develop severe pancreatitis[26].

RATIONALE FOR FLUID RESUSCITATION

Management of AP revolves around supportive care, adequate nutrition, and intravenous hydration. The rationale for hydration is based on the need to resolve the hypovolemia that occurs secondary to vomiting, reduced oral intake, third space extravasation, respiratory losses and diaphoresis. In addition, early hydration provides macrocirculatory and microcirculatory support to prevent the cascade of events leading to pancreatic necrosis[34].

Correction of hypovolemia, as assessed by changes in hematocrit, BUN and serum creatinine, has been documented to limit necrosis and improve outcome. Hemoconcentration, as a marker of hypovolemia and severity of pancreatitis, has been studied since the 1960s[35]. A hematocrit of ≥ 44%-47% on admission combined with failure of a decrease in the hematocrit at 24 h was reported as the best risk factor for development of necrosis[36]. Wu *et al*[37] showed that early hemoconcentration was associated with increased mortality only among hospital transferred cases, and not among non-transferred cases. This difference could be due to variations in the early management of the studied cases,

Table 1 Recommendations in various reviews for fluid resuscitation in acute pancreatitis

Ref.	Type	Conclusion
Tenner *et al*[7], 2004	Review	250-500 mL/h or more for 48 h
Whitcomb *et al*[79], 2006	Review	Fluid bolus: maintain hemodynamics
		Later: 250-500 mL/h
Otsuki *et al*[91], 2006	Review	60-160 mL/kg per day
		1/3 to 1/2 to be given in 6 h
Forsmark *et al*[96], 2007	Review	Use crystalloids first,
		Use colloids if hematocrit < 25% or albumin < 2 g/dL
Pandol *et al*[78], 2007	Review	Severe volume depletion: 500-1000 mL/h; reduce later
Nasr *et al*[8], 2011	Review	20 mL/kg (1-2 L) in emergency;
		150-300 mL/h (3 mL/kg per hour) for 24 h
Trikudanathan *et al*[49], 2012	Review	Aggressive fluid resuscitation in patients with AP needs to be initiated with therapeutic intent
Haydock *et al*[49], 2013	Review	Lack of quality evidence to guide most basic aspects of FT providing the equipoise necessary for further RCTs
Wu *et al*[31], 2013	Review	Institutional protocols must be developed to help ensure adequate fluid resuscitation, particularly in initial 24 h

AP: Acute pancreatitis; FT: Fluid therapy; RCT: Randomized controlled trial.

Table 2 Summary of available studies to date on controlled fluid therapy

Ref.	Year	Type of study (sample size)	Conclusion
Mao *et al*[16]	2010	RCT (*n* = 155)	Rapid hemodilution increases incidence of sepsis within 28 d and in-hospital mortality. Hematocrit should be maintained between 30% and 40% in acute response stage
Mao *et al*[17]	2009	RCT (*n* = 76)	Controlled fluid resuscitation offers better prognosis in patients with severe volume deficit within 72 h of severe acute pancreatitis onset
Eckerwall *et al*[15]	2006	Retrospective cohort (*n* = 99)	Patients receiving 4000 mL or more of fluid in first 24 h developed more respiratory complications
Madaria *et al*[14]	2011	Retrospective cohort (*n* = 247)	Administration of > 4.1 L but not < 3.1 L was significantly associated with more local and systemic complications
Kuwabara *et al*[75]	2011	Retrospective (*n* = 9489)	Fluid volume during first 48 h was higher in patients requiring ventilation and higher mortality in acute pancreatitis

RCT: Randomized controlled trial.

further emphasizing the fact that fluid resuscitation should be instituted early. Similarly, changes in BUN and creatinine from baseline are indicative of intravascular volume depletion and both these markers are used to predict outcome. Monitoring of these parameters can gauge the effectiveness of initial resuscitative measures[38-43]. These parameters can, therefore, be used to optimize goal-directed resuscitation.

Microcirculatory disturbances in AP are different from simple hypovolemia of trauma or bleeding, as they are caused by SIRS with overexpression of inflammatory mediators which injure the endothelium and increase capillary permeability, leading to fluid sequestration and capillary leak syndrome[44]. Thus, the purpose of effective fluid resuscitation in severe AP is not only to replenish the blood volume but also to stabilize the capillary permeability, modulate the inflammatory reaction, and sustain intestinal barrier function[44].

A number of animal studies have shown the benefit of fluid resuscitation in AP, using both colloids and crystalloids. Juvonen *et al*[45] used a pig model of AP to show that signs of splanchnic hypoperfusion could

be prevented with fluid resuscitation. In that study, the authors measured splanchnic perfusion by local pCO_2 gap, oxygen delivery and consumption, lactate production, and blood flow. On the other hand, Niederau *et al*[46] used a mouse model of AP to show that subcutaneous administration of fluid could normalize hemoconcentration and improve survival. High volume crystalloid infusion with Ringer's lactate has also been shown to improve pancreatic microcirculation in a canine model[47] and with balanced salt solution in a rat model[48].

Which patients require fluid resuscitation?

The primary aim of fluid therapy is to limit or prevent pancreatic necrosis. Any patient with AP has the potential to progress to severe disease. Patients with mild interstitial pancreatitis are commonly kept under observation in the emergency room, and once their pain settles they can be discharged. Patients with underlying comorbidities would, however, require closer observation. The Revised Atlanta guidelines[3] allow for patients to be triaged and evaluated for severity. Patients with moderate and severe AP require observation for organ failure and local or systemic
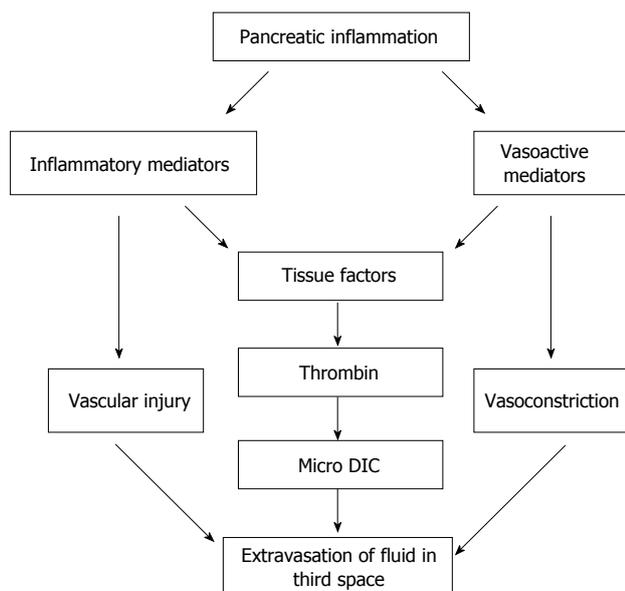
Figure 1 Pathophysiology of fluid depletion in pancreatitis.

complications, and should be started on fluid therapy. It must be recognized that at the time of first interface with the patient it may not be possible to gauge severity, which may evolve over the next 24-48 h.

Choice of fluid

Two types of fluids used frequently are colloids and crystalloids. Commonly used colloids are various formulations of dextran, hetastarch and albumin. Colloids are considered superior to crystalloids in optimization of the hemodynamic response[49]. They also have better retention in the intravascular compartment because of their larger size[34]. As they remain within the lumen, despite increased vascular permeability, they help to maintain better circulatory flow. In addition, they contribute to the correction of hypovolemia because of their osmotic effect in drawing fluid from the interstitium to the vascular compartment. However, colloids can cause intravascular volume overload, hyperoncotic renal impairment, coagulopathy, and anaphylactic reaction[50].

Commonly used crystalloids are normal saline (NS), lactated Ringer's (RL) and Ringer's ethyl pyruvate, with hypertonic saline being the so-called "new kid on the block". Crystalloids are distributed in both the plasma and the interstitial compartments, and large spaces are therefore required to restore the circulation. Infusion of large amounts of crystalloids could lead to pulmonary edema[48]. Hypertonic saline effectively reduces the volume of isotonic fluid resuscitation, thereby reducing the risk of pulmonary edema[51]. However, there is a potential risk of central pontine myelinolysis with aggressive hypertonic saline therapy[52].

The ideal fluid for resuscitation in AP is yet to be determined. The choice is primarily between a colloid and a crystalloid. Variability in the results of initial studies with fluid resuscitation could be attributed to different types of fluids used. Colloids have been shown to be superior to crystalloids in animal experiments[34], which may be due to the fact that they are not as permeable to leakage in pancreatic microcirculation as crystalloids. By remaining in the lumen, circulatory blood flow is better maintained and inflammatory mediators are less likely to reach the acinus when colloids are used[34].

Most of the animal studies on fluid resuscitation have used colloid solutions and found them to be better than crystalloids. Schmidt *et al*[53] showed intra-aortic bolus infusion of high-dose dextran to be better than LR and NS with regards to necrosis and mortality in a rat model of pancreatitis. Other researchers have also shown beneficial effects of different dextran preparations[47,54-57]. Klar *et al*[55] quantified the effect of dextran on pancreatic microcirculation during experimental acute biliary pancreatitis and showed that stabilization of microcirculation was accomplished in 6 h. In yet another study, mortality was reduced from 60% in RL controls to 10% and 0% in groups treated with DEX-70 and DEX-160 respectively[57].

The use of colloids in humans has involved various preparations of dextran and albumin. In a phase I human study involving 13 patients with severe non-biliary pancreatitis, after hemodilution with dextran, progression of pancreatic necrosis was seen in 15% and mortality was seen in only 7.7%[55]. In another human study, 32 patients with severe AP were treated with 500-1000 mL dextran 40 and dexamethasone (0.5-1 mg/kg) daily along with standard therapy for one week. Twenty-seven patients improved, 5 required surgery and 4 ultimately succumbed to death. The authors concluded that short period use of dexamethasone can inhibit inflammatory mediators while dextran helps in correcting microcirculatory disturbances[58].

Hydroxyethyl starch (HES) is another colloid fluid that can preserve systemic oxygenation in patients with capillary leak. While it has been shown to reduce the risk of intra-abdominal hypertension in severe AP[59], no impact on organ failure and mortality has been observed. In a recent study comparing NS (group 1), HES and NS (group 2) and HES, NS and glutamine (group 3), Zhao *et al*[44] showed that patients in groups 2 and 3 had decreased rates of organ failure and infection. However, a Cochrane review has concluded that HES may actually increase mortality in critically ill patients[60].

In contrast to the animal studies, there is more data on beneficial effects of crystalloids in humans. Du *et al*[59] compared RL with a combination of RL and HES. The combination showed better results on intra-abdominal pressure in AP but no effect was seen on organ failure, in-hospital stay or mortality. A recent randomized controlled trial by Wu *et al*[61] compared NS *vs* RL as resuscitation fluid in AP and reported dampening of systemic inflammation after 24 h in subjects resuscitated with RL. A significant reduction in the prevalence of SIRS and levels of C-reactive protein was found in the RL group as compared to the NS group. This pilot trial

of RL as the primary resuscitation fluid has become a landmark study for the potential utility of RL as a resuscitation fluid in early treatment of AP.

Recently, hypertonic saline was found in various animal studies to be useful in management of AP by modulation of cytokine expression[43,62,63]. It has been shown to significantly facilitate pancreatic microcirculation and to have favorable effects on cardiac contractility and peripheral tissue perfusion[51]. Aerosolized hypertonic saline has also been shown to reduce lung injury as well as the risk of lung edema, due to the lower volume used[51]. However, safety of hypertonic saline for volume repletion needs to be established as adverse potential effects, such as renal failure, have been documented in burn patients[64].

A combination of crystalloids and colloids has been suggested by some researchers to be better than either alone[17,43]. Moreover, some researchers have suggested using a ratio of 1:3 for colloids and crystalloids respectively[65]. In a recent study of 47 patients with AP, Chang *et al*[66] showed that a crystalloid to colloid ratio of 1.1-3.0 was superior to a ratio of < 1.5 or > 3.

Other solutions used in fluid resuscitation are a cell-free hemoglobin-based oxygen carrier[67], a combination of isovolemic hemodilution with HES and a plasmatic oxygen carrier[68], 6% hydroxyethyl starch and furosemide[69]. A recent Chinese study demonstrated the effects of 6% HES and furosemide on the outcome of AP, but only 4% of the patients with Ranson's score of 3 or more and 7% with a Balthazar computed tomography (CT) score D developed pancreatic necrosis and organ dysfunction[69]. Another study demonstrated the effect of fresh frozen plasma (FFP) in AP and observed that the ability of FFP to reduce the fall in serum α-2 macroglobulin may have therapeutic implications in early treatment of AP[70].

In summary, there is a lack of high level evidence to guide the choice of fluid in AP. Crystalloids are recommended by the American Gastroenterological Association, and colloids (packed red blood cells) are considered in cases of low hematocrit (< 25%) and low serum albumin (< 2 g/dL). Among the crystalloids, RL solution is preferred over NS[71]. However, there is an urgent need of studies on this issue.

Volume and rate of fluid resuscitation: What is aggressive?

Aggressive fluid resuscitation was defined by the Mayo Clinic group to constitute ≥ 33% of the total volume in 72 h of infusion performed in the first 24 h[72]. Chinese researchers have used a more objective criteria of 15 mL/kg per hour infusion as aggressive resuscitation, as compared to controlled resuscitation, which they defined as 5-10 mL/kg per hour[17]. Although recommendations by various authors and reviews have suggested intensive fluid resuscitation, it was two retrospective studies from Mayo Clinic[72,73] that defined aggressive fluid resuscitation.

In the first study, 28 patients in the non-aggressive group experienced higher mortality (17.9%) than the 17 patients in the aggressive group (0% mortality)[73]. In the second study, out of a total 73 patients, the 31 who were given non-aggressive fluid resuscitation had higher SIRS scores[38]. In a separate retrospective study, Wall *et al*[74] observed the mean rate of hydration fluid being higher (234 mL/h in first 6 h and 221 mL/h in first 12 h) in patients treated in 2008 as compared to those treated in 1988 (194 mL/h in first 6 h and 188 mL/h during first 12 h) with a decrease in mortality and necrosis in 2008 as compared to 1998.

There are, however, other studies suggesting that aggressive hydration may be associated with increased morbidity and mortality[14,15]. de-Madaria *et al*[14] analyzed 247 patients prospectively who were divided into three groups depending on the fluid received in the initial 24 h. Administration of > 4.1 L during the initial 24 h was found to be associated with persistent organ failure and acute collections, while administration between 3.1 and 4.1 L was associated with an excellent outcome. Eckerwall *et al*[15] reported that patients receiving > 4.0 L in the first 24 h developed more respiratory complications (66% *vs* 53%, *P* < 0.001) than those receiving less than 4.0 L/24 h. The latter group had decreased need for ICU care, as well. A Japanese study analyzed the demographics of fluid resuscitation in 9489 patients and found that those with higher volume infused in the first 48 h had higher respiratory complications and higher mortality[75].

Mao *et al*[17] found higher complication rates and mortality in patients given aggressive hydration (15 mL/kg per hour) when compared to those given controlled hydration (5-10 mL/kg per hour). Another study by Mao *et al*[16] showed that survival rate in the slow hemodilution group (84.7%) was better than that in the rapid hemodilution group (66%). These authors also found that rapid hemodilution can increase the incidence of sepsis within 28 d.

Concept of controlled hydration

Since there has been a negative side effect reported for aggressive fluid resuscitation, some practitioners have proposed a more controlled fluid resuscitation rather than overzealous fluid therapy. A Chinese study of 83 patients confirmed the observations that survival rates improved significantly by controlling fluid resuscitation and preventing sequestration of body fluids[18].

It has been pointed out that there are an equal number of studies in favor of aggressive and non-aggressive fluid resuscitation[9]. One can argue that aggressive resuscitation restores the intravascular compartment depleted by "third spacing" and results in more effective end-organ tissue perfusion and reverses pancreatic ischemia[76]. Those in favor of non-aggressive hydration suggest that by the time we intervene in patients with AP, pancreatic necrosis is already non-reversible and aggressive fluid therapy will only lead to respiratory

failure and increased intra-abdominal pressure, *etc.*[76]. Therefore, a "controlled" resuscitation aimed at reversing hypotension, and being able to maintain effective mean arterial pressure (MAP) and urine output > 0.5 mL/kg, is the best bet[76].

How much fluid is sequestrated?

Recommendations from various societies and groups have suggested aggressive fluid resuscitation in AP[7,34,71,77]; however, these recommendations were not based on any hard data. Although it is well known that some patients with AP have an increased need for fluid therapy, it is not clear who should get fluids aggressively. In a recent study, de-Madaria *et al*[14] prospectively calculated fluid sequestration in AP by subtracting fluid output from fluid intake in the first 48 h. Fluid intake included all intravenous fluids, oral fluids and blood, while fluid output included volumes of urine, stool, vomitus, as well as insensible losses (10 mL/kg per day). The median fluid sequestration in the first 48 h after hospitalization was 3.2 L (1.4-5 L), 6.4 L (3.6-9.5 L) in those without necrosis and those with necrosis, and 7.5 L (4.4-12 L) in those with persistent organ failure. Fluid sequestration correlated with age, alcohol as an etiology, hematocrit, and SIRS score. This study provided the first data for the possible fluid requirement in AP and determinants of aggressive fluid resuscitation. In a retrospective study performed in 1974, Ranson *et al*[29] had reported a mean fluid sequestration at 48 h of 3.7 L *vs* 5.6 L in mild and severe pancreatitis. The data from de-Madaria *et al*[14] will be useful in planning future prospective trials.

Rate of fluid replacement: need for bolus

Tenner had given the first recommendation of rate of fluid replacement as 250-500 mL/h for 48 h in 2004[7]. Subsequently, Pandol *et al*[78] suggested that in cases of severe volume depletion fluids can be infused at 500-1000 mL/h followed by 300-500 mL/h to replace non-pancreatic fluid loss. However, these recommendations would amount to up to 6-12 L of fluid in the first 24 h, which is beyond the volumes used in different studies, both prospective and retrospective. Even in studies in which early aggressive hydration had been used, the total volume in the first 24 h did not generally exceed 6 L[72,73]. Whitcomb[79] was the first one to suggest that fluid should be started as a bolus, followed by maintenance. Nasr *et al*[8] suggested that a bolus of 20 mL/kg should be given in the emergency room, followed by 150-300 mL/h (3 mL/kg per hour) for 24 h. In their recent study on goal-directed fluid resuscitation, Wu *et al*[61] used a bolus of 20 mL/kg, followed by another bolus and 3 mL/kg per hour (210 mL/h in a 70 kg patient) in fluid refractory and 1.5 mL/kg per hour (105 mL/h) without another bolus in fluid responsible patients. Thus, it seems reasonable to start with a bolus and follow it up with maintenance fluid infusion. This recommendation is close to the guidelines for septic shock, which recommend an initial bolus with crystalloids at the rate of 1000 mL/h,

with a minimum of 30 mL/kg, which also includes use of vasopressor epinephrine to maintain adequate blood pressure. However, more data is needed from prospective studies to reach consensus on this issue. It must be mentioned here that the Fluid Expansion as Supportive Therapy (FEAST) trial of fluid bolus in African children with severe infection had actually resulted in greater mortality[80].

Does one-size fit all?

A recent review has pointed out there are reports favoring and opposing early aggressive fluid resuscitation[9]. It has also been acknowledged that benefits of early aggressive therapy have not been confirmed prospectively[14]. Early aggressive therapy with predetermined fluid infusion for the first 24 h to 48 h is based on the assumption that fluid sequestration and hence fluid requirement over this time frame is the same for all the patients. However, de-Madaria *et al*[14] have reasoned that patients who develop local complications after admission are prone to more fluid sequestration, so they require more fluids. They suggest that fluid resuscitation and its replacement is a dynamic process and patients with local complications should receive heightened fluid intake on the second and third days of admission.

All the recommendations and guidelines presume that patients would report within 24 h of onset of pancreatitis. Since this is not the case, generally, especially in referral centers, there is a need to take this factor into account as, the longer the delay in hospitalization, the more established the hemodynamic alterations become.

Special situations

Patients with co-morbidities like renal failure, cardiac compromise and pulmonary disease need special attention. As per the Revised Atlanta classification system, comorbidities are important determinants of severity of AP[3]. Moreover, occurrence of organ failure as a consequence of AP calls for special attention in such patients. Management of such patients may be extrapolated from the Surviving Sepsis Campaign guidelines for management of septic shock[81]. Issues in such patients that need special considerations include the rate of fluid transfused, use of additional agents like vasopressors, and the need for specific monitoring. There may be a need to restrict fluid infusion in those patients who have renal dysfunction or cardiac dysfunction. As in patients with sepsis, vasopressor therapy is required to maintain perfusion pressure in the face of hypotension, even when hypovolemia has not yet been treated. Some patients require vasopressors to achieve a minimum perfusion pressure. In patients with sepsis, titration of norepinephrine to a MAP as low as 65 mmHg has been shown to preserve tissue perfusion[82]. Norepinephrine is the recommended vasopressor in such situations[82]. Management of adult respiratory distress syndrome is as per standard guidelines[82]. Recently, extracorporeal life support for acute pancreatitis-induced acute respiratory

distress syndrome has been shown to be beneficial[83].

Role of continuous hemodiafiltration or continuous veno-venous hemofiltration

Since hypercytokinemia is believed to be pivotal in pathophysiology of severe AP, the role of continuous hemodiafiltration (CHDF) has been investigated for removal of pro-inflammatory cytokines[84]. CHDF with a polymethylmethacrylate (PMMA) membrane removes various cytokines from the bloodstream and is widely used in Japan for blood purification therapy in patients with morbid conditions and is thought to prevent organ failure. In a retrospective review of 10 years' experience, Pupelis *et al*[85] summarized the clinical application of continuous veno-venous hemofiltration (CVVH) in patients with AP and concluded that it may help in balancing fluid replacement and removal of cytokines from the blood and tissue compartments. Early application of CVVH helped in achieving cumulative negative fluid balance starting at day 5 in patients with AP and intra-abdominal hypertension (IAH) in one study[85]. It has been investigated for decreasing intra-abdominal pressure (IAP) in patients with AP[86-88], and Japanese guidelines suggest blood purification therapy as recommendation C in severe AP which may prevent OF but not the mortality[89]. Xu *et al*[90] recently demonstrated a decrease in tumor necrosis factor-alpha (TNF-α) levels after initiation of CVVH; they also showed a positive correlation between blood levels of TNF-α and IAP. Pupelis *et al*[91] have documented that use of CVVH as a part of conservative treatment of AP decreases the need for surgical intervention from 41% to 19%. Even though neither CHDF nor CVVH has been recommended so far in the treatment of severe AP, due to non-availability of quality evidence, it is expected to contribute in the improvement of AP outcome in the future[85].

RESUSCITATION GOALS

Fluid resuscitation needs to be monitored by periodic assessment of cardiovascular, renal and pulmonary functions, as well as electrolyte imbalances. A drop in hematocrit and BUN has often been recommended as a marker of hemoconcentration correction. Hematocrit has been used for over 50 years to guide fluid replacement in critically ill patients; in AP, it has been identified as a marker that correlates with development of pancreatic necrosis[36,92]. Failure of hematocrit decrease has been correlated with increased necrosis and poor outcome. Similarly, elevated BUN has been used as a marker of severe disease, and the failure of BUN to decrease has been reported in patients with increased necrosis. In a recent study, Wu *et al*[61] used BUN to define fluid responsiveness. At 8-12 h after of the start of resuscitation, if the BUN level remained unchanged or increased from its previous value, participants were considered refractory. The authors used this key parameter as a target for goal-directed fluid therapy.

The aim of monitoring is accurate prediction of the response to fluids before volume expansion occurs. The classic static parameters for monitoring are central venous pressure (CVP), pulmonary artery occlusion pressure (PAOP) and MAP. However, these may be fallacious in patients receiving mechanical ventilation and in those with IAH[93]. The dynamic parameters measuring cardiac response to changes in preload, such as systolic volume variation and pulse pressure variation (PPV), can be used in patients on mechanical ventilation but requires caution in those with IAH[93]. In a recent study of an experimental model of AP, Trepte *et al*[94] showed that goal-directed fluid resuscitation using stroke volume variation (SVV) led to better survival, tissue oxygenation, and microcirculatory perfusion when compared to CVP and MAP measurements. However, in an accompanying editorial, it was pointed out that in an experimental setting resuscitation can be started immediately after induction of AP, but in clinical practice patients usually reach the hospital with already established dramatic fluid shifts with hypovolemia, increased hematocrit, and IAH[95]. Moreover, the use of SVV indispensably requires controlled mechanical ventilation, which is not a realistic scenario at admission of such patients. Also SVV/PPV are less useful in patients with IAH[95]. Huber *et al*[95] suggest that strategies combining volumetric and dynamic parameters (where applicable) with functional tests (passive leg raising and expiratory occlusion), and also taking into account IAP and abdominal perfusion pressure, need to be evaluated prospectively.

Though CVP is often used in critical care units to monitor fluid resuscitation, it is not an ideal end point in patients with AP because there is a disconnect between the intracellular space and the intravascular compartment[23]. A high CVP in patients with severe AP may indicate intravascular repletion when the intracellular compartment is actually under-resuscitated.

Urine output measurement remains an easy end point to monitor. Adequate urine output confirms adequate intravascular repletion. While urine output of > 0.5 mL/kg per hour was considered adequate by a technical review[96], the Japanese recommend a urine output greater than 1 mL/kg per hour[5]. However, recent studies have used > 0.5 mL/kg per hour as the end point of fluid resuscitation[61]. In a recent editorial, the same has also been recommended[76]. Similar to other critical illnesses, serum lactate levels have also been recommended to monitor resuscitation in AP[97]; however, there is no evidence of its application in patients with AP.

Because of the compounding influence of mechanical ventilation, IAH, pleural effusion and mediastinal edema, the pressure-based parameters may not accurately reflect adequacy of fluid replacement in AP. Therefore, newer hemodynamic measurements, such as the intrathoracic blood volume index, global end diastolic volume index and extravascular lung water index, have been suggested[49]. However, there is need to have more data and the fact that these measures are invasive makes it less

Table 3 Authors' recommendations for fluid replacement in predicted severe or severe acute pancreatitis

Parameter	Recommendation
Fluid resuscitation	Necessary: the earlier the resuscitation, the better the outcome
Type of fluid	Colloids and/or crystalloids:
	Among crystalloids, lactate Ringer's better than normal saline
	Use colloids especially when albumin < 2.0 g/dL or hematocrit < 35%
Amount of fluid	Total fluid in first 24 h: between 3 and 4 L, Not to exceed 4 L
Rate of infusion	Initial bolus 1000 mL over one hour followed by 3 mL/kg per hour (200 mL/h) for 24-48 h
Monitoring	Urine output > 0.5 mL/kg/h, hematocrit = 25% to 35%, drop in BUN
	CVP: Not good for monitoring due to third space loss and hypoalbuminemia
Duration of resuscitation	24-48 h, until signs of volume depletion disappear

BUN: Blood urea nitrogen; CVP: Central venous pressure.

likely that they will be used routinely.

Responsiveness to fluid therapy

Responsiveness to fluid resuscitation can be assessed by measurement of hematocrit, BUN, urine output or intrathoracic volume index, among other parameters. As highlighted above, Wu *et al*[61] have used BUN to classify patients as responsive or refractory to fluid resuscitation. A simple maneuver of leg elevation has been suggested to determine fluid responsiveness in patients having spontaneous breathing. An increase in ≥ 10% in cardiac output during the first 60-90 s of leg elevation can predict response to fluid replacement[98].

Monitoring for intra-abdominal hypertension

Severe AP can become complicated by IAH, which can become worsened by aggressive fluid resuscitation. In healthy individuals, IAP ranges from 0 mmHg to 5 mmHg and varies with the respiratory cycle. IAH is a sustained increase in IAP > 12 mmHg. Abdominal compartment syndrome is a sustained increase in IAP > 20 mmHg, with new onset organ failure with or without a low abdominal perfusion pressure. The incidence of IAH in patients with severe AP is approximately 60% to 80%[97]. IAH is usually an early phenomenon, partly related to the effects of the inflammatory process causing retroperitoneal edema, fluid collection, ascites and ileus and is partly iatrogenic, resulting from aggressive fluid resuscitation[97]. It is associated with impaired organ dysfunction, especially of the cardiovascular, respiratory and renal systems[98].

Since it contributes to fluid non-responsiveness and carries a high surgical mortality, IAH should be monitored in all patients with severe AP. Presence of intra-abdominal pressure > 16 mmHg makes it difficult to correctly interpret results of passive leg raising, as

well as of other static and dynamic pressure parameters. Abdominal perfusion pressure can serve as a good marker and resuscitation end point in patients with IAH[99]. Maintaining abdominal perfusion pressure above 50-60 mmHg is recommended in order to facilitate adequate perfusion of abdominal organs[97]. There is some evidence that use of HES resuscitation reduces the risk of IAH in severe AP[59]; however, HES may actually increase mortality in critically ill patients[60].

Frequency of monitoring

As the pathogenesis of AP is a dynamic process, with local and systemic complications compounding the clinical management, it is imperative to monitor fluid resuscitation closely. After an initial bolus of 20 mL/kg or 1000 mL over one hour, followed by controlled fluid infusion for the first 24 h, the patients should be evaluated closely in a high dependency unit. Hemodynamically, the goals chosen to monitor should be assessed at 8 h to 12 h intervals. Wu *et al*[61] evaluated their patients every 8 h for the first 24 h. Although there are no guidelines on this issue, it seems logical to follow the practice of Wu *et al*[61].

CONCLUSION

Fluid resuscitation has emerged as a key therapeutic strategy in patients with acute pancreatitis. It has to be acknowledged that fluid resuscitation in AP is a complex process, with need to take into account the dynamics of fluid sequestration during different stages of the disease. Current knowledge suggests that controlled fluid resuscitation (3.0-4.0 L/24 h) should be started after a bolus infusion of 20 mL/kg (1000 mL over one hour). Among the different fluids, lactated Ringers' is the one recommended by most guidelines. There is a need to carry out fluid resuscitation with a goal-directed strategy, with urine output > 0.5 mL/kg and a drop in BUN as simple goals. There is a need for randomized controlled trials to generate data on all the three issues addressed (rate, type and end points of fluid resuscitation) before definite guidelines can be framed. The authors have summarized recommendations about fluid therapy in predicted severe or severe AP (Table 3).

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2014 ADVANCES IN PANCREATITIS

Chronic pancreatitis: A surgical disease? Role of the Frey procedure

Alexandra Roch, Jérome Teyssedou, Didier Mutter, Jacques Marescaux, Patrick Pessaux

Alexandra Roch, Jérome Teyssedou, Didier Mutter, Jacques Marescaux, Patrick Pessaux, Digestive and Endocrine Surgery, Nouvel Hôpital Civil, University of Strasbourg, 67000 Strabourg, France

Alexandra Roch, Jérome Teyssedou, Didier Mutter, Jacques Marescaux, Patrick Pessaux, Institut de recherche en cancérologie de l'appareil digestif (IRCAD), 67000 Strasbourg, France

Alexandra Roch, Jérome Teyssedou, Didier Mutter, Jacques Marescaux, Patrick Pessaux, IHU-Strasbourg, Minimally-Invasive Image-Guided Surgical Institute, University of Strasbourg, 67000 Strabourg, France

Author contributions: All the authors contributed to this paper.

Correspondence to: Patrick Pessaux, MD, PhD, Professor, Head of the Hepatobiliary and Pancreatic Surgical Unit, Digestive and Endocrine Surgery, Nouvel Hôpital Civil, University of Strasbourg, 1 place de l'hopital, 67000 Strasbourg, France. patrick.pessaux@chru-strasbourg.fr

Telephone: +33-3-69551563

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Abstract

Although medical treatment and endoscopic interventions are primarily offered to patients with chronic pancreatitis, approximately 40% to 75% will ultimately require surgery during the course of their disease. Although pancreaticoduodenectomy has been considered the standard surgical procedure because of its favorable results on pain control, its high postoperative complication and pancreatic exocrine or/and endocrine dysfunction rates have led to a growing enthusiasm for duodenal preserving pancreatic head resection. The aim of this review is to better understand the rationale underlying of the Frey procedure in chronic pancreatitis and to analyze its outcome. Because of its hybrid nature, combining both resection and drainage, the Frey procedure has been conceptualized based on the pathophysiology of chronic pancreatitis. The short and long-term outcome, especially pain relief and quality of life, are better after the Frey procedure than after any other surgical proce-
dure performed for chronic pancreatitis.

Core tip: The management and the treatment of chronic pancreatitis are challenging. Many surgical procedures were described with 2 different types of concepts: resection *vs* drainage. The Frey procedure is an association of these 2 concepts. This manuscript contains the most recent data about the technique, the short and long-term outcomes of this technique. In addition, there is a review of the literature of series comparing this technique with the other surgical procedures.

INTRODUCTION

Chronic pancreatitis is a progressive inflammatory disease characterized by debilitating pain and pancreatic insufficiency (nutritional deficiency and glucose deregulation)[1,2]. The enormous personal and socioeconomic impact comprises impairment of quality of life, inability to work and even shortening in life expectancy[3]. Although medical treatment and endoscopic interventions are primarily offered to patients with chronic pancreatitis[4,5], approximately 40% to 75% will ultimately require surgery during the course of their disease[6,7].

Although pancreaticoduodenectomy has been considered the standard surgical procedure for patients with chronic pancreatitis because of its favorable results

on pain control, its high postoperative complication and pancreatic exocrine or/and endocrine dysfunction rates[8,9] have led to a growing enthusiasm for duodenal preserving pancreatic head resection[10,11]. When in 1987 Frey *et al*[12] described a novel hybrid procedure combining local resection of the head of the pancreas and longitudinal pancreatico-jejunostomy, surgeons favorably welcomed it because of its technical feasibility and low surgical risk. Since 1987, numerous studies have analyzed the short and long-term outcome following the Frey procedure and have compared it to other surgical procedures commonly performed for chronic pancreatitis. The aim of this review is to better understand the rationale underlying of the Frey procedure in chronic pancreatitis and to analyze its outcome.

WHY CAN CHRONIC PANCREATITIS BE CONSIDERED A SURGICAL DISEASE?

Mechanisms of pain in chronic pancreatitis

Although pain is the most common symptom (85% of patients)[2] in chronic pancreatitis, its mechanism remains unclear and debated[13-15]. Several concepts have been hypothesized and pain probably results from a combination of them. The intraductal and interstitial hypertension theory is similar to a compartment syndrome[16,17]. Increased ductal pressure related to duct stricture or calculi and intraparenchymal hypertension as a result of fibrosis and edema can activate intrapancreatic nociceptors. The neurogenic theory focuses on intrapancreatic neural damage[18]. Inflammatory mediators from infiltrating lymphocytes are responsible for increased signals along the axons of pain-sensitive neurons, which ultimately can result in a "centrally sensitized" pain state[19]. Traditionally, the head of the pancreas is called the "pacemaker" of pain in chronic pancreatitis. It is often enlarged and can be replaced by an inflammatory mass that can lead to common bile duct or duodenal stenosis[20]. Another explanation to this pain is the compression of adjacent organs by a pseudocyst.

Indications for surgery

Surgical management is usually offered to patients after medical treatment and endoscopic intervention have failed[4,5], and is considered the last option of this step-up approach[21]. Medical treatment for pain related to chronic pancreatitis usually fails, as narcotic dependency occurs in most patients[11]. Longitudinal studies have shown that 40% to 75% of all patients with chronic pancreatitis will require surgery in the course of the disease[7]. The main indications for surgery are intractable pain, non-resolving common bile duct or duodenal stenosis and suspicion of malignancy. The objective of surgery is to relieve intractable pain while preserving pancreatic endocrine and exocrine functions.

Rationale for surgery in chronic pancreatitis

First, surgery has been proved superior to endoscopic treatment in 2 main randomized controlled trials[22,23]. Moreover, several studies have suggested that surgery early in the course of chronic pancreatitis is beneficial in terms of pain control and pancreatic function[21,24]. One experimental and three clinical observational cohort studies have concluded that surgery, especially drainage procedures, can delay the natural course and progressive loss of pancreatic function in chronic pancreatitis. In an experimental model of early *vs* late surgical drainage in pigs, early surgery resulted in less pathological cell damage and better exocrine function[25]. When Nealon *et al*[26] compared the outcomes of conservative treatment *vs* surgery, they reported a delay in pancreatic function impairment after surgical treatment. They concluded that early operative drainage should be performed before the pancreas shows morphological and functional irreversible damage. Ihse *et al*[27] also have recommended surgical treatment to be performed before nutritional or metabolic disorders develop.

Prolonged periods of pain can be associated with peripheral and central nerve sensitization, leading to a permanent state of pain impossible to reverse[19]. A recent observational study suggests that longstanding disease is associated with poor pain control after surgical intervention[28]. In 266 consecutive patients undergoing surgery for chronic pancreatitis, surgery after 3 years of onset of symptoms was independently associated with impaired pain relief and increased rate of endocrine pancreatic insufficiency. A small pilot trial randomized 32 patients with early stage chronic pancreatitis and dilated pancreatic duct between upfront surgical drainage and conservative approach[29]. Significant pain relief was observed in 94% patients in the surgical group compared to 13% patients in the conservative group. New onset pancreatic insufficiency was significantly less frequently observed in the early surgical group compared conservative group. Despite the evidence suggesting a benefit of early surgery, most patients are still managed by a conservative step-up approach. To evaluate the benefits, risks and costs of early surgical intervention, the Dutch Pancreatitis Study Group is currently conducting a multicentric randomized controlled trial (the Early Surgery *vs* Optimal Current Step-up Practice for Chronic Pancreatitis trial)[21].

The role of chronic pancreatitis as a risk factor for pancreatic carcinogenesis has been supported by numerous studies since 1993[30-32]. Lowenfels *et al*[30] published an international cohort study of 2015 patients that reported a cumulative risk of pancreatic cancer in subjects with chronic pancreatitis of 1.8% after 10 years and 4%, after 20 years with a standardized incidence ratio of 14.4. A recent multicentric Japanese study[33] of 506 patients found that the incidence of pancreatic cancer was significantly lower in patients who underwent surgery for chronic pancreatitis than in patients who had a conservative treatment (0.7% *vs* 5.1%, *P* = 0.03, HR = 0.11). Although this study shows a protective effect of surgery in the development of pancreatic cancer from chronic pancreatitis, the exact mechanism remains unclear probably through reduction in pancreatic inflammation.

FREY PROCEDURE: SURGICAL TECHNIQUE

Rationale for the Frey procedure

Based on the pathophysiological mechanisms described above[13-19], two main surgical procedure types have been described in patients with chronic pancreatitis: drainage and resection procedures[11,34,35]. Until the late 80s, pancreaticoduodenectomy was the resection procedure of choice for "head-dominant" disease[11]. The Frey procedure was first described in 1987 by Frey *et al*[12] and combines partial resection of the head of the pancreas (resection) with lateral pancreatico-jejunostomy (drainage). The rationale for this hybrid procedure[12, 36-38] is that it improves the overall pancreatic ductal drainage by decompressing both the duct of Santorini and ducts in the uncinate process. It also allows removal of calculi. Moreover, the partial pancreatic head resection removes what is thought to be the "epicenter" of chronic pain and can relieve symptoms related to ductal stricture.

The Frey procedure was originally applied to patients with an enlarged fibrotic head of the pancreas and an associated dilated main pancreatic duct. It has since then been described in various indications, including patients who have had prior lateral pancreatico-jejunostomy (Puestow or Partington and Rochelle procedures) with no relief of symptoms[38].

Surgical technique

Through a bilateral subcostal incision and after exposure of the pancreas (Kocher maneuver), the main pancreatic duct is located using a syringe aiming toward the tail of the pancreas[12,36-40]. The pancreatic duct is then opened longitudinally (the incision in the tail of the pancreas is extended to within 1-2 cm of the distal portion of the gland and the incision in the head to within 1 cm of the inner aspect of the duodenum). When the main pancreatic duct is exposed, it can be inspected and all calculi removed. The head of the pancreas is partially cored-out while preserving a rim of pancreatic tissue along the inner aspect of the duodenum (to allow blood supply to the duodenum from superior and inferior pancreaticoduodenal arteries), along the pancreatic medial margin (to avoid injuring the superior mesenteric/portal vein) and posteriorly (between the head excavation and the uncinate process and vena cava). During the local excision of the head of the pancreas, the intrapancreatic portion of the common bile duct is freed from inflamed and fibrotic periodical tissue. In about 70% of cases, resection of the fibrotic pancreatic parenchyma is sufficient to relieve a common bile duct stricture. If the obstruction cannot be relieved, a choledocho-duodenostomy or a choledocho-jejunostomy can be performed. The cored-out head of the pancreas and the open main duct are drained into Roux-en-Y limb of jejunum. The Roux-en-Y limb is passed through the transverse mesocolon to lie over the pancreas. A two-layer pancreatico-jejunostomy is performed. The gastrointestinal tract continuity is restored by and end-to-side jejuno-jejunostomy. Owing to the increased risk of pancreatic cancer in patients with chronic pancreatitis, the cored tissue from the pancreatic head is routinely sent for pathological analysis.

Technical key points

Compared to other surgical procedures (especially pancreaticoduodenectomy and Beger procedure), the Frey procedure is easier to perform by avoiding the transsection of the pancreas neck over the superior mesenteric/portal vein.

Although Frey *et al*[37-39] analyzed the relation between weight of the cored pancreatic head tissue and pain relief, this amount of tissue depends on the size of the head of the pancreas, which is highly variable. Some studies suggested that a mean volume percent of head mass resected between 60% and 65% allowed better pain relief. Extensive pancreatic head excision should not be performed as it may lead to increased parenchymal loss and ultimately pancreatic exocrine insufficiency.

Current data suggest that the Frey procedure in small duct chronic pancreatitis is associated with a significantly increased operative time[41]. Difficulty in locating the main pancreatic duct contributes to the delay and intra operative ultrasound in those cases proves useful[42].

Because the Frey procedure can be technically challenging due to major chronic inflammation, it is traditionally performed as an open surgery. Surgeons from John Hopkins recently published a case report describing a total laparoscopic Frey procedure for chronic pancreatitis caused by recurrent pancreatic ductal stones[43]. The laparoscopic approach confers the benefits of magnified visualization while reducing the rate of postoperative wound infection, incisional hernia, bowel obstruction and pain[44]. As laparoscopic Frey procedure is very demanding, the selection of patients that can benefit from it is very important. This approach will less likely be offered to obese patients, as visualization can be impaired by retroperitoneal fat. Similarly, this approach does not fit patients with a highly vascular head of the pancreas because of the increased risk of bleeding.

RESULTS OF THE FREY PROCEDURE

Complications

The Frey procedure can be performed with low mortality (< 2%). The published complication rates range from 7% to 42%[45-50]. The most common complications include hemorrhage, pancreatic fistula and intra-abdominal abscess. Arterial bleeding is the major life-threatening complication (2%-3%). It can occur several days from surgery after erosion of per pancreatic vessels by pancreatic fluid from an anastomotic leakage, or due to the rupture of a pseudoaneurysm[41,49]. Late complications rate after the Frey procedure is high, probably because of comorbidities (alcohol, smoking) in most patients with chronic pancreatitis. The main medical complication is pulmonary infection and/or insufficiency[50]. In 2006, Pessaux *et al*[49]

recommended preoperative respiratory physiotherapy for all patients before the Frey procedure to avoid postoperative respiratory complications.

Short and long-term outcome

Exocrine insufficiency has been described in up to 79% of patients following the Frey procedure, whereas de novo diabetes occurs in only 8% to 34% of patients[45-50].

Keck *et al*[47] showed that 62% of patients were completely pain free 5 years after the Frey procedure. Similarly, Negi *et al*[51] showed that the Frey procedure led to significant and sustained complete or partial pain relief in 75% over a median follow-up of 6 years. This study suggests that the Frey procedure significantly decreases the severity of recurrent exacerbations and also the number of acute episodes requiring hospital readmission. Falconi *et al*[52] reported up to 90% of partially or completely pain-free patients after the Frey procedure. Hildebrand *et al*[53] showed that the indices for global quality of life and for physical and emotional status increased after the Frey procedure.

Factors predicting outcome

Ten to 20% of patients demonstrate persistent pain after the Frey procedure[44-50]. Several risk factors for poor pain relief have been described in the literature, with controversial results[54]. In 1999, Frey and Amikura[38] found that chronic narcotic use, multiple abdominal interventions before pancreatic surgery were associated with poor outcome, whereas Riediger *et al*[55] found that preoperative exocrine insufficiency and postoperative surgical complications were the strongest predictors of poor pain relief. In an Indian study[41], preoperative use of opiates, continuous pattern of pain and postoperative complications were significant predictive factors of failure to achieve complete pain relief after surgery. However, even patients who used opiate medication preoperatively benefited from surgery (significant reduction in pain score, number of pain exacerbation and hospital readmissions). These results suggest that preoperative narcotic use should not be considered a contraindication to the Frey procedure although patients should be referred for surgery early in the course of chronic pancreatitis before drug addiction becomes an issue.

The correlation between main pancreatic duct diameter and pain relief after the Frey procedure remains debated[38,56,57]. A recent study from John Hopkins showed that the degree of pancreatic fibrosis correlated with the resolution of pain in a series of 35 patients treated with the Frey procedure[58]. Their results suggest that pain in patients with extensive pancreatic fibrosis is significantly better relieved by the Frey procedure than in patients with mild or minimal fibrosis. They implied that patients with mild or minimal fibrosis may respond more favorably to other procedures such as total pancreatectomy with islet auto-transplantation. Determination of pancreatic fibrosis extent preoperatively, thanks to improving imaging technologies, might be an important variable to choose the surgical procedure more likely to achieve pain relief. They also found an association between ductal dilation $\geq$ 4 mm and better pain relief. However, they believe that the influence of main pancreatic duct diameter on outcome following the Frey procedure may be biased, as ductal dilation is usually the consequence of progressive fibrosis. In these cases, an alternative could be an extended drainage by "V-shaped excision" advocated by Izbicki *et al*[59] and Yekebas *et al*[60] with a partial head resection. This technique seems to be a secure and effective approach for small duct chronic pancreatitis achieving significant improvement in quality of life and pain relief.

Comparison Frey vs other surgical procedures for chronic pancreatitis

Frey procedure *vs* pancreaticoduodenectomy: Operation time is shorter with the Frey procedure, with lower intraoperative blood loss and perioperative transfusion requirements[61]. Chiang *et al*[62], in a prospective study comparing the Frey procedure to pancreaticoduodenectomy found no difference in mortality, morbidity, pain relief or improvement in pancreatic function 3 and 6 mo after surgery. One randomized controlled trial including 61 patients compared the outcome of pancreaticoduodenectomy and Frey procedure[63]. In this trial (follow-up of 2 years), Izbicki *et al*[63] found better results after Frey procedure regarding quality of life, although pain relief was similar after both procedures. Additionally, the rate of complications after the Frey procedure was significantly lower than after pancreaticoduodenectomy (19% *vs* 53%). Farkas *et al*[64] supported those results concluded that the Frey procedure led to better long-term quality of life. In the long-term follow-up study (mean of 7 years) published by Strate *et al*[65], there was no difference between Frey and pancreaticoduodenectomy regarding late mortality, survival rate, exocrine and endocrine insufficiency (although the rates of new onset diabetes after both procedures were twice higher than preoperatively) and need for reintervention. The initial favorable results of quality of life and pain after Frey procedure still existed but were not statistically significant. Interestingly, Aspelund *et al*[66] found however a significantly lower incidence of new onset diabetes after the Frey procedure (8%) than after pancreaticoduodenectomy (25%). A recent randomized controlled trial presented at the European Surgical Association in 2013 reported the 15-year follow-up of the Frey procedure *vs* pancreaticoduodenectomy for chronic pancreatitis[67]. They concluded that long-term pain relief was comparable after both surgical procedures but the quality of life was better after the Frey procedure. Moreover, mean survival was significantly shorter after pancreaticoduodenectomy because of a higher delayed and long-term mortality rate. Regarding weight gain and work rehabilitation, the Frey procedure also showed better outcome than pancreaticoduodenectomy[53] (Table 1).

Frey procedure *vs* Beger procedure: A randomized controlled trial comparing the Frey procedure with Be-

Table 1 Main studies comparing surgical procedures for chronic pancreatitis

Ref.	Year	Study design	Comparison	Median follow-up (in months)
Izbicki *et al*[63]	1998	Retrospective	Frey *vs* PPPD	24
Aspelund *et al*[66]	2005	Retrospective	Frey *vs* PD	36
Hildebrand *et al*[53]	2010	Retrospective	Frey *vs* PD	50
Farkas *et al*[64]	2006	Prospective	Frey *vs* PPPD	24
Chiang *et al*[62]	2007	Prospective	Frey *vs* PD	6
Strate *et al*[65]	2008	Prospective	Frey *vs* PPPD	84
Bachmann *et al*[67]	2013	Prospective	Frey *vs* PD	180
Izbicki *et al*[46]	1995	Prospective	Frey *vs* Beger	18
Strate *et al*[68]	2005	Prospective	Frey *vs* Beger	104
Keck *et al*[47]	2010	Retrospective	Frey *vs* Beger	20.6

PPPD: Pylorus-preserving pancreaticoduodenectomy; PD: Pancreaticoduodenectomy.

ger procedure[46] found that the Frey procedure was associated with a lower complication rate (9% *vs* 15%). In the 8-year follow-up study published by Strate *et al*[68] in 2005, both procedures showed equivalent mortality, pain relief, exocrine/endocrine insufficiency, rate of reintervention and quality of life. Similarly, a study by Keck *et al*[47] including 92 patients showed a trend toward better pain control but similar pancreatic insufficiency rates and weight gain after the Frey procedure when compared to the Beger procedure.

In conclusion, because of its hybrid nature, combining both resection and drainage, the Frey procedure has been conceptualized based on the pathophysiology of chronic pancreatitis. The short and long-term outcome, especially pain relief and quality of life, are better after the Frey procedure than after any other surgical procedure performed for chronic pancreatitis.

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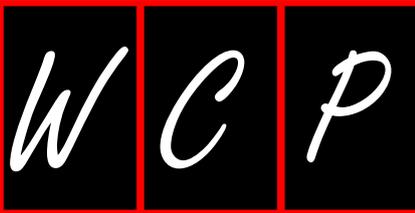
Prevention of post-ERCP pancreatitis

Lin-Lee Wong, Her-Hsin Tsai

Lin-Lee Wong, Her-Hsin Tsai, Department of Gastroenterology, Castle Hill Hospital, HEY NHS Trust and Hull York Medical School, Cottingham HU165JQ, United Kingdom

Author contributions: Wong LL performed the searches and prepared the original draft; Tsai HH edited and supplemented the manuscript.

Correspondence to: Dr. Her-Hsin Tsai, Department of Gastroenterology, Castle Hill Hospital, HEY NHS Trust and Hull York Medical School, Hull Royal Infirmary, Anlaby Road., Hull, East Riding of Yorkshire, Cottingham HU165JQ, United Kingdom. hhtsai@doctors.org.uk

Telephone: +44-1482-875875 Fax: +44-1482-622159
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Abstract

Post-procedure pancreatitis is the most common complication of endoscopic retrograde cholangio pancreatography (ERCP) and carries a high morbidity and mortality occurring in at least 3%-5% of all procedures. We reviewed the available literature searching for "ERCP" and "pancreatitis" and "post-ERCP pancreatitis". in PubMed and Medline. This review looks at the diagnosis, risk factors, causes and methods of preventing post-procedure pancreatitis. These include the evidence for patient selection, endoscopic techniques and pharmacological prophylaxis of ERCP induced pancreatitis. Selecting the right patient for the procedure by a risk benefits assessment is the best way of avoiding unnecessary ERCPs. Risk is particularly high in young women with sphincter of Oddi dysfunction (SOD). Many of the trials reviewed have rather few numbers of subjects and hence difficult to appraise. Meta-analyses have helped screen for promising modalities of prophylaxis. At present, evidence is emerging that pancreatic stenting of patients with SOD and rectally administered nonsteroidal anti-inflammatory drugs in a large unselected trial reduce the risk of post-procedure pancreatitis. A recent meta-analysis have demonstrated that rectally administered indomethecin, just before or after ERCP is associated with significantly lower rate of pancreatitis compared with placebo [OR = 0.49 (0.34-0.71); *P* = 0.0002]. Number needed to treat was 20. It is likely that one of these prophylactic measures will begin to be increasingly practised in high risk groups.

Core tip: Select patients carefully, and give high risk patients rectal indomethacin.

INTRODUCTION

Pancreatitis is the most common complication of endoscopic retrograde cholangio pancreatography (ERCP) and carries a high morbidity and mortality[1,2]. There is a 3%-5% incidence of this complication occurring, as shown in various large clinical studies[2-4]. A systematic survey of 21 studies involving 16855 patients (1987-2003) found a 3.5% occurrence of post-ERCP pancreatitis. 0.4% of patients had severe pancreatitis with 0.11% deaths[5].

Predicting pancreatitis after ERCP can be very difficult but there have been numerous studies that have identified factors that increase the risk for post-ERCP pancreatitis. These can have a cumulative effect when multiple factors are present. There are multiple procedures and pharmacological interventions that have been studied to prevent post-ERCP pancreatitis. This article describes these some of these interventions and includes the latest studies.

Table 1 Consensus definition of post-endoscopic retrograde cholangio pancreatography pancreatitis

Severity of pancreatitis	Definition
Mild	Clinical pancreatitis, amylase at least 3 × normal > 24 h after procedure, requiring unplanned admission or prolongation of planned admission to 2-3 d
Moderate	Hospitalisation of 4-10 d
Severe	Hospitalisation of > 10 d, haemorrhagic pancreatitis, pancreatic necrosis or pseudocyst, or need for intervention (percutaneous drainage or surgery)

DIAGNOSIS OF POST-ERCP PANCREATITIS

Post-ERCP pancreatitis is defined as acute pancreatitis occurring following an ERCP procedure. This consists of the development of new pancreatic-type abdominal pain associated with hyperamylasemia of three times the upper-limit of normal, occurring 24 h after an ERCP requiring hospital admission. Severity of post-ERCP pancreatitis is graded based on length of hospital admission and need for intervention. It can be divided into mild, moderate and severe (Table 1), based on a consensus definition[6].

Freeman *et al*[1] studied the complication rate that occurred in 2347 patients undergoing endoscopic biliary sphincterotomy. Acute pancreatitis occurred in 127 patients (5.4%). Mild post-ERCP pancreatitis occurred in 53 (2.3%), moderate in 65 (2.8%) and severe in 9 (0.4%). Of the latter, one died of retroperitoneal perforation, one required percutaneous drainage of a pseudocyst and three required surgical drainage.

RISK FACTORS FOR POST-ERCP PANCREATITIS

Many studies have looked into factors that increase the risk of post-ERCP pancreatitis. These can be divided into patient-related risk factors, endoscopist-related risk factors and procedure-related risk factors. Table 2 summarises the general consensus of risk factors for post-ERCP pancreatitis[3,7,8]. These factors should alert the endoscopist to take special precautions in preventing post-ERCP pancreatitis[9]. In addition, there is a cumulative effect for patients with multiple risk factors. For example, a young woman with suspected sphincter of Oddi dysfunction, normal bilirubin, difficult cannulation and absence of bile duct stones has an associated increased risk of pancreatitis of 40%[10,11].

There are other factors that have been identified which require further studies. One retrospective study identified taking pancreato-toxic drugs (oestrogen, azathioprine, valproic acid, mesalazine, morphine derivatives and prednisone) increased the occurrence of post-ERCP pancreatitis (OR = 3.7)[12].

Another retrospective study of 506 patients identified angiotensin receptor blockers and smoking as

Table 2 Risk factors for post-endoscopic retrograde cholangio pancreatography pancreatitis

Risk factors for post ERCP pancreatitis	
Patient-related factors	Younger age
	Female sex
	Normal serum bilirubin
	Recurrent pancreatitis
	Prior ERCP-induced pancreatitis
	Sphincter of Oddi dysfunction
Endoscopist-related factors	Difficult cannulation
	Pancreatic duct injection
	Sphincter of Oddi manometry
	Precut sphincterotomy
	Pancreatic sphincterotomy
	Minor papilla sphincterotomy
Procedure-related factors	Trainee involvement in procedure

ERCP: Endoscopic retrograde cholangio pancreatography.

independent risk factors for post-ERCP pancreatitis[13] whereas a recent case-control study of 6505 patients identified smoking and chronic liver disease as factors that reduced the risk of post-ERCP pancreatitis[14].

Testoni *et al*[7] conducted a large prospective multicentre trial (total of 3635 ERCP procedures) and showed that the rate of post-ERCP pancreatitis did not differ between high- and low-volume centres (3.9% *vs* 3.1%). However, the high-volume centres treated a larger proportion of patients at high-risk of pancreatitis and did a significantly greater number of difficult procedures. In another large multicentre prospective trial (2347 patients), case volume did not affect incidence of pancreatitis although the multivariate model indicated low case volume was independently associated with higher overall rate of complications[1].

Operator experience has been difficult to demonstrate as a risk factor for post-ERCP pancreatitis due to the heterogeneity of studies with variable case volume and case mix. One French study showed no risk associated with operator inexperience[14].

In the multivariate analysis of a randomised controlled multicentre study by Cheng *et al*[8], trainee involvement in the procedure was found to be a risk factor (OR = 1.5) for development of post-ERCP pancreatitis.

Biliary stenting was found to be an independent risk factor for pancreatitis in a single-centre prospective study by Wilcox *et al*[15]. The commonest indication for stent placement was pancreaticobiliary malignancy (37% of patients). Another retrospective study on patients undergoing ERCP for malignant biliary obstruction found the frequency of post-ERCP pancreatitis was significantly higher with placement of self-expanding metal stents compared with a plastic stent[16].

MECHANISM OF POST-ERCP PANCREATITIS

There are various mechanisms proposed in the pathogenesis of post-ERCP pancreatitis[17,18]. These include: (1)

mechanical injury from instrumentation of papilla and pancreatic duct; (2) thermal injury following application of electrosurgical current during biliary or pancreatic sphincterotomy; (3) hydrostatic injury - following injection of contrast medium into the pancreatic duct of from infusion of water or saline solution during sphincter manometry; (4) chemical or allergic injury following injection of contrast medium into the pancreatic duct; (5) enzymatic injury with intraluminal activation of proteolytic enzymes; and (6) infection from contaminated endoscope and accessories.

Preventive measures are aimed at interrupting the cascade of events resulting in the premature activation of proteolytic enzymes, autodigestion and impaired acinar secretion with subsequent clinical manifestations of local and systemic effects of pancreatitis[17].

PREVENTION OF POST-ERCP PANCREATITIS

ERCP technique

Cannulation: Various methods to ease cannulation of the bile duct and reduce trauma have been studied with view of reducing the risk of post-ERCP pancreatitis.

In general, guidewire technique to facilitate bile duct cannulation has been shown to improve primary biliary duct cannulation but incidence of post-ERCP pancreatitis has not been consistently shown to be reduced by this technique.

In a meta-analysis of five randomised controlled trials (RCTs), guidewire cannulation was shown to lower post-ERCP pancreatitis (rates 0%-3%) compared to standard contrast-injection method (rates 4%-12%) and increase primary cannulation rates compared to the standard method (OR = 2.05)[19].

A Cochrane meta-analysis of 12 RCTs (3450 patients) similarly found that post-ERCP pancreatitis incidence was lower in the wire-guided cannulation (WGC) group (3.5%) compared to contrast-assisted cannulation technique (6.7%) and primary cannulation rates were higher in the WCG group (84% *vs* 77%, RR = 1.07). However, WGC may not prevent post-ERCP pancreatitis in patients with suspected Sphincter-of-Oddi dysfunction and unintentional pancreatic duct guidewire cannulation[20].

In contrast, a recent crossover multicentre randomised controlled trial involving 322 patients compared wire-guided biliary cannulation with conventional cannulation technique - the trial found that the incidence of post-ERCP pancreatitis was similar in both groups (6.1% *vs* 6.3%, *P* = 0.95). Primary biliary cannulation rate was similar for both groups as well (83% *vs* 87%)[21].

Another prospective trial involving 1249 patients did not find any significant difference in the rates of post-ERCP pancreatitis with the guidewire technique compared with sphincterotome and contrast injection method[22].

Many advanced endoscopists use a hybrid of the two techniques (wire probes with minimal contrast to outline distal duct course) which avoid dissections or passage of the guidewire out of a side branch of the pancreatic duct. This hybrid technique however has not been formally evaluated[23].

Electrocautery: Thermal injury following application of electrosurgical current during biliary or pancreatic sphincterotomy is thought to contribute to causing post-ERCP pancreatitis. A number of studies have been conducted to compare pure cut current with blended current and bipolar *vs* monopolar electrocautery. These have produced mixed results. A meta-analysis of four trials (total: 804 patients) comparing pure current to mixed current in patients who underwent sphincterotomy found no significant difference in the rates of pancreatitis. Pure current was however associated with more episodes of bleeding, primarily mild bleeding[24]. The use of sequential combination of pure cut and blended current for sphincterotomy was studied in 142 patients - this did not change the rate of post-ERCP pancreatitis but did cause less visible bleeding than pure cut alone[25].

Pancreatic stenting: Pancreatic duct obstruction or impaired pancreatic drainage from papillary oedema or spasm of the sphincter of Oddi has been postulated to cause post-ERCP pancreatitis[17]. Numerous studies have looked into the prophylactic placement of a pancreatic stent to prevent post-ERCP pancreatitis. Due to their variability in indications for stenting, interventions and outcome measures - comparisons and conclusions can be difficult. A few trials have shown that pancreatic stent insertion reduces the rate and severity of post-ERCP pancreatitis after difficult cannulation, needle-knife precut, biliary sphincterotomy for sphincter of Oddi dysfunction (SOD) and manometry, pancreatic sphincterotomy, endoscopic ampullectomy and endoscopic balloon dilation[26-33].

A recent meta-analysis of randomised controlled trials (RCTs) comparing pancreatic stent placement and the subsequent incidence of post-ERCP pancreatitis enrolled 14 studies (total: 1541 patients). This found that pancreatic stent placement was associated with significant reduction of post-ERCP pancreatitis (RR=0.39, 95%CI: 0.29-0.53, *P* < 0.001) as compared with no stent placement. Subgroup analysis demonstrated that pancreatic stent placement was effective for both high-risk and mixed case groups[34].

Another meta-analysis by Choudhary *et al*[35] analysed eight RCTs (656 patients) and this showed that prophylactic pancreatic stents decreased the odds of post-ERCP pancreatitis (OR = 0.22; 95%CI: 0.12-0.38, *P* < 0.01) with an absolute risk difference of 13%.

Pancreatic stenting comes with some limitations. It is associated with complications such as stent-related ductal injury and strictures[36]. Many endoscopists and assistants are unfamiliar with the placement of pancreatic stents. In addition, unsuccessful stent placement can itself be associated with a risk of pancreatitis. Freeman *et al*[37] conducted a prospective study of 225 high risk ERCPs.

Table 3 Pharmacological agents studied according to postulated mechanism of action

Postulated mechanism of action	Agents
Interruption of inflammatory cascade	NSAIDs, steroids, interleukin-10, allopurinol, adrenaline spray, pentoxifylline, platelet-activating factor-acetylhidrolase, semapimod, aprepitant, risperidone
Reduction of pancreatic enzyme secretion	Octreotide, somatostatin, calcitonin
Inhibition of protease activity	Gabexate mesilate, heparin, ulinastatin, nafamostat, magnesium sulphate
Reduction of Sphincter-of-Oddi pressure	Nitroglycerin, nifedipine, botulinum toxin, lidocaine, secretin, phosphodiesterase inhibitor type 5
Prevention of infection	Antibiotics
Anti-oxidants	Beta-carotene, N-acetylcysteine, sodium selenite
Anti-metabolites	5-fluorouracil

NSAIDs: Non-steroidal anti-inflammatory drugs.

Pancreatitis occurred in two out of three (66.7%) patients in whom stent insertion failed *vs* 32 of 222 (14.4%) patients with successful insertion ($P = 0.06$).

Follow-up evaluation is necessary to ensure passage or removal of stent and placement can be technically difficult. The optimal timing for stent placement and duration for stent to remain in place is unknown. There is also variability in the type of stent used[17,33]. Short 5 French stents are easier to deploy and are more likely to migrate spontaneously compared with long 3 French stents. However, they do not confer a benefit in terms of pancreatitis risk reduction. The optimal duration for stents to remain in place is unknown. Chahal *et al*[38] compared the outcomes of a short straight 5 French stent without an inner flange with an unflanged long single pigtail 3 French stent. They found a significantly higher placement failure rate in the 3 French group (8.3% *vs* 0%, $P = 0.0003$), a higher spontaneous dislodgement rate in the 5 French group (98% *vs* 88% for 3 Fr, $P = 0.0001$) and a non-significant higher pancreatitis rate (14% *vs* 9%, $P = 0.3$).

Pharmacological prophylaxis

Since the introduction of ERCP, numerous studies have been carried out in the pursuit to discover the most effective pharmacological prophylactic agent against post-ERCP pancreatitis. These were done based on the postulated mechanisms of action through which post-ERCP pancreatitis occurred (Table 3)[39,40].

Interruption of inflammatory cascade (anti-inflammatory): Non-steroidal anti-inflammatory drugs: non-steroidal anti-inflammatory drugs (NSAIDs) have been studied for their inhibitory properties on phospholipid A2 (PLA2) and prostaglandins, which lead to interruption of the inflammatory cascade of acute pancreatitis[41]. A Finnish group, Mäkelä *et al*[42] studied the in-vitro inhibition of PLA2 in acute pancreatitis by 17 different pharmacological agents. They found that indomethacin

was the most potent of the agents in inhibiting PLA2 activity in the serum from patients with acute pancreatitis followed by diclofenac.

Murray *et al*[43] conducted a prospective, randomised, double-blind controlled trial involving 220 patients. In the twenty-four patients (11%) who developed acute pancreatitis, 7 had received 100mg diclofenac suppository given immediately after ERCP *vs* 17 who received placebo ($P < 0.05$). They concluded that rectal diclofenac given immediately after ERCP can reduce the incidence of acute pancreatitis.

Since then, three meta-analysis have been published, analysing the effect of NSAIDs in preventing post-ERCP pancreatitis. The results of each meta-analyses are as follows: (1) Elmunzer *et al*[44]: Four RCTs (912 patients) evaluating rectal NSAIDs (indomethacin or diclofenac) administration in the peri-procedure period were analysed. This found a significant reduced incidence of pancreatitis with pooled relative risk of 0.36. The pooled number needed to treat with NSAIDs to prevent one episode of pancreatitis was 15; (2) Dai *et al*[45]: Six RCTs (1300 patients) were analysed. These included the 4 RCTs in the above-mentioned meta-analysis as well as two additional trials. Two trials used rectal diclofenac, three used rectal indomethacin and one used oral diclofenac. The risk of pancreatitis was lower in the NSAID group than in the placebo group (OR = 0.46, $P < 0.0001$)[45]; and (3) Ding *et al*[46]: Meta-analysis of ten RCTs (2269 patients) showed that NSAIDs decreased the overall incidence of post-ERCP pancreatitis (RR = 0.57, $P = 0.007$) with an absolute risk reduction of 5.9% and number needed to treat: 17. In addition, NSAIDs use decreased the incidence of moderate to severe post-ERCP pancreatitis (RR = 0.46, $P = 0.002$). This meta-analyses included studies that were heterogenous in NSAIDs-type (indomethacin, diclofenac or valdecoxib) and route of administration. Rectal administration of NSAIDs was associated with a decreased risk of post-ERCP pancreatitis in all six trials that used this route while the other routes studied in 4 studies (oral, intramuscular, intravenous and intraduodenal) were not.

Rectal administration is the most effective route for NSAIDs in post-ERCP prevention. This is postulated to be due to wider bioavailability compared to oral route (with significant first-pass metabolism) and the quicker peak plasma NSAIDs concentrations (30 min for rectal route *vs* 2 h for oral route)[47,48].

All the trials showed no adverse effects from NSAIDs administration to patients. However, limitations to the meta-analyses were differences in pharmacological manipulation (timing, route of administration and choice of drug), inconsistent use of pancreatic stenting, inclusion of both high-risk and low-risk patients and differences in ERCP procedures (*e.g.*, number of cannulations, number of pancreatic duct injections, whether sphincterotomy was performed). In addition, different definitions of pancreatitis were used [some used 4 × upper limit of normal (ULN) hyperamylasemia while some used 3 × ULN with abdominal pain][44,45]

The latest multi-centre trial by Elmunzer *et al*[49] was carried out using a randomised, placebo-controlled and double-blind method. This compared rectal indomethacin *vs* placebo immediately after ERCP. A total of 602 patients were enrolled of which 82% were high-risk (suspected sphincter of Oddi dysfunction). Rectal indomethacin was found to significantly reduce the incidence of post-ERCP pancreatitis (9.2% *vs* 16.9%, *P* = 0.005).

A recent meta-analysis have demonstrated that rectally administered indomethacin, just before or after ERCP is associated with significantly lower rate of pancreatitis compared with placebo [OR = 0.49 (0.34-0.71); *P* = 0.0002]. Number needed to treat was 20. Moreover they found that in subgroup analysis, the difference remained unchanged for average-risk population [OR = 0.49 (0.28-0.85); *P* = 0.01] or in preventing severe PEP [OR = 0.41 (0.21-0.78); *P* = 0.007][50]. The European Society of Gastrointestinal Endoscopy published guidelines in 2010 with grade A recommendation for the administration of rectal diclofenac 100 mg or indomethacin immediately before or after ERCP as post-ERCP prophylaxis[51]. The United States and United Kingdom however have not yet come to a consensus regarding this.

The available evidence suggests that prophylactic rectal administration of NSAIDs should be used in high-risk patients due to its marked reduction in incidence post-ERCP pancreatitis. This will result in substantial medical and cost benefits.

Other anti-inflammatory agents: Glucocorticoids have been evaluated as a potential prophylactic agent in a few studies (intravenous and oral). Initial promising reports have been followed by five prospective controlled trials which have demonstrated its inefficacy in preventing post-ERCP pancreatitis[52-58]. Finally, a meta-analysis of six randomised controlled trials using intravenous or oral corticosteroids (total: 2448 patients) demonstrated that prophylactic corticosteroids did not reduce the incidence of post-ERCP pancreatitis[59].

Interleukin-10 is an anti-inflammatory cytokine that has been shown to limit the severity of acute pancreatitis in animal models. One initial study (144 patients, placebo-controlled) found the incidence of pancreatitis was reduced by a single IV dose given 30 min before ERCP (8% *vs* 24% in placebo)[60]. It was also effective for high-risk patients. However, two subsequent placebo-controlled trials (total 505 patients) did not demonstrate any efficacy[61,62].

Allopurinol has been studied for its inhibitory properties on oxygen-derived free radicals. Trials studying the effect of allopurinol on post-ERCP pancreatitis prevention have revealed conflicting results. Subsequent two meta-analyses of 10 RCTs (1554 patients and 1730 patients respectively) have concluded that allopurinol does not reduce post-ERCP pancreatitis and should be not recommended as a prophylactic agent[63,64].

Other agents studied (Adrenaline spray, pentoxifylline, platelet-activating factor acetylhidrolase, semapimod, aprepitant and risperidone) have either revealed discordant results or no effect on preventing post-ERCP pancreatitis[64-73].

Reduction of pancreatic secretion: Somatostatin and its synthetic analogue, octreotide are potent inhibitors of exocrine secretion of the pancreas. Various studies have been conducted using different dosing regimes (< 6 h, ≥ 12 h or bolus). Andriulli *et al*[73] conducted a meta-analyses (16 studies) which concluded that somatostatin was ineffective in preventing post-ERCP pancreatitis. Two further controlled trials by Lee *et al*[74] and Chan *et al*[75] revealed conflicting results. Similar mixed results were found in studies using octreotide[76-78]. Therefore, somatostatin and octreotide are currently not recommended as a prophylactic agents.

Calcitonin has been studied and not been shown to have any prophylactic effect on pancreatic enzymes or complication rate[79,80].

Inhibition of protease activity: Protease inhibitors prevent activation of trypsin which is involved in the cascade of events leading to acute pancreatitis. Gabexate mesilate, nafamostat and ulinastatin have been studied in numerous studies. However, results of the trials have been conflicting. Some trials showed a benefit in reducing post-ERCP pancreatitis while others did not show any effect, especially in high-risk patients.

Seta *et al*[81] published a meta-analysis on 18 studies (4966 patients) evaluating the efficacy of protease inhibitors. This found that protease inhibitors showed a small risk reduction in ERCP-associated pancreatitis with high number needed to treat (34.5). Overall, the analysis concluded that there was no solid evidence to support the use of protease-inhibitors to prevent ERCP-associated complications.

A more recent meta-analysis by Yuhara *et al*[82] compared the effects of protease inhibitors and NSAIDs. This included 19 studies (nafamostat mesilate, *n* = 4 studies, NSAIDs, *n* = 7 studies and gabexate mesilate, *n* = 6 studies and ulinastatin, *n* = 2 studies). This found that nafamostat mesilate and NSAIDs had solid evidence for preventing post-ERCP pancreatitis (RR = 0.41 and RR = 0.58 respectively) while gabexate and ulinastatin were not associated with decreased risk of post-ERCP pancreatitis. These findings differed from the former meta-analysis by Seta *et al*[81] which did not distinguish between gabexate mesilate, ulinastatin and nafamostat mesilate.

Heparin has been studied for its anti-inflammatory properties with discordant results. A meta-analysis of four trials (1438 patients) demonstrated no benefit for prophylactic heparin in prevention of post-ERCP pancreatitis[83].

Magnesium sulphate (intravenous) is currently being studied as a calcium-antagonist and hence, a prophylactic agent against post-ERCP pancreatitis[84].

Reduction of sphincter-of-oddi pressure: Reducing sphincter of Oddi pressure would theoretically prevent development of post-ERCP pancreatitis.

Initial trials studying the effect of GTN (transdermal

or sublingual) showed promise[85,86] but three subsequent randomised trials demonstrated no significant preventive effect on post-ERCP pancreatitis[87-89].

Numerous other drugs have been studied with disappointing or conflicting results. These include nifedipine, botulinum toxin, lidocaine and phosphodiesterase inhibitor type 5[90].

Secretin causes relaxation of the Sphincter of Oddi and increases pancreatic secretion. Studies on secretin have revealed mixed results. In a German randomised trial studying the influence of secretin and gabexate-mesilate on ERCP-related complications, secretin was shown to have no effect on ERCP-induced hyperamy-lasemia[91]. On the other hand, Jowell *et al*[92] conducted a single-centre randomised placebo-controlled trial (869 patients) using intravenous secretin (16 μg) administered immediately before ERCP *vs* placebo. Secretin was found to decrease the incidence of pancreatitis (8.7% *vs* 15.1% in the placebo group, $P = 0.004$). Subgroup analysis revealed that secretin was highly protective against post-ERCP pancreatitis for patients undergoing biliary sphincterotomy (6/129 *vs* 32/132, $P < 0.001$).

Prevention of infection

Antibiotics: One old controlled study has evaluated the role of antibiotics on post-ERCP pancreatitis and found no effect on its incidence[93]. Another prospective randomised controlled trial involving 315 patients demonstrated that 2 g of ceftazidime administered intravenously 30 min before ERCP significantly reduced the incidence of post-ERCP pancreatitis (2.6% *vs* 9.4% in the control group, $P = 0.009$). However, this study was deemed of low-methodological quality due to the unclear allocation concealment (the control group received "no antibiotics" in place of placebo). Further studies are required before antibiotics can be recommended as a prophylactic agent against post-ERCP pancreatitis[94,95].

Anti-oxidants: Oxidant stress may be involved in the pathogenesis of post-ERCP pancreatitis. N-acetylcysteine and sodium selenite have both been studied in randomised controlled trials and was shown to not reduce the incidence of post-ERCP pancreatitis[96]. Beta-carotene was studied in a double-blind trial and did not reduce incidence of pancreatitis between the treatment and placebo group. However, there was some postulated protective effect of treatment with beta-carotene seen as there were no patients with severe pancreatitis, as compared to the placebo group (2.22%)[97].

A recent meta-analysis looked at of 11 randomised trials (3010 patients) using N-acetylcystein, selenite, beta-carotene, allopurinol and pentoxifylline. This concluded that anti-oxidant supplementation shows no beneficial effect on the incidence and severity of post-ERCP pancreatitis[98].

CONCLUSION

Selection of patients, good technique, and good aftercare remain the primary prevention of post-ERCP pancreatitis. Currently, rectal NSAIDs are the only pharmacological agents that have been shown to reduce the incidence of post-ERCP pancreatitis in especially in high-risk patients and is gaining wider acceptance. The other agents (protease inhibitors and anti-secretory agents) require larger multi-centre randomised trials that can control for multiple variables. ERCP techniques should be adapted according to the risk-profile of the patient. Guidewire technique eases primary biliary cannulation but has not been shown to reduce incidence of post-ERCP pancreatitis. Patient selection and stratifying risk in individual patients is vital in preventing post-ERCP pancreatitis. Manipulation should be minimised in high-risk cases. In addition, pancreatic stenting should be used in high-risk patients, particularly young female patients with suspected sphincter of Oddi dysfunction, difficult cannulation or history of post-ERCP pancreatitis.

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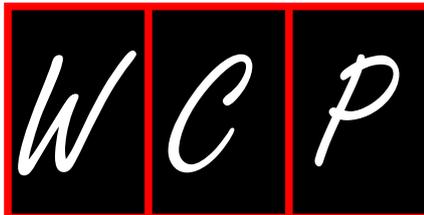
WJGP 5th Anniversary Special Issues (3): Pancreatitis

Pathophysiology of autoimmune pancreatitis

Raffaele Pezzilli, Nico Pagano

Raffaele Pezzilli, Nico Pagano, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, 40138 Bologna, Italy

Author contributions: Pezzilli R and Pagano N acquired, analyzed and interpreted the data in the literature; they drafted the manuscript and approved the version of the paper to be published.

Correspondence to: Raffaele Pezzilli, MD, Department of Digestive Diseases and Internal Medicine, Sant'Orsola-Malpighi Hospital, Via Massarenti 9, 40138 Bologna, Italy. raffaele.pezzilli@aosp.bo.it

Telephone: +39-51-6364148 Fax: +39-51-6364148

Abstract

Autoimmune pancreatitis (AIP) is a recently discovered form of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically. International consensus diagnostic criteria have been developed, and the clinical phenotypes associated with the histopathologic patterns of lymphoplasmacytic sclerosing pancreatitis and idiopathic duct-centric pancreatitis should be referred to as type 1 and type 2 AIP, respectively. Most importantly, in type 1 AIP, the pancreatic manifestations are associated with other extrapancreatic disorders, resembling an immunoglobulin G4 (IgG4)-related disease. In addition, the pancreas of a patient with AIP is often infiltrated by various types of immune cells; the cluster of differentiation (CD) 4 or CD8 T lymphocytes and IgG4-bearing plasma cells have been found in the pancreatic parenchyma and other involved organs in AIP and factors regulating T-cell function may influence the development of AIP. From a genetic point of view, it has also been reported that *DRB1*0405* and *DQB1*0401* mutations are significantly more frequent in patients with AIP when compared to those with chronic calcifying pancreatitis, and that only *DQB1*0302* had a significant association with the relapse of AIP. Finally, it has been found that the polymorphic genes encoding cytotoxic T lymphocyte-associated antigen 4, a key negative regulator of the T-cell immune response, are associated with AIP in a Chinese population. Even if these data are not concordant, it is possible that physiological IgG4 responses are induced by prolonged antigen exposure and controlled by type 2 helper T cells. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and cellular immune responses.

Core tip: Autoimmune pancreatitis (AIP) is a recently discovered form of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically. Two types of AIP have been recognized: type 1 (usually associated with other extrapancreatic disorders) and type 2. The pancreas of a patient with AIP is often infiltrated by various types of immune cells, including cluster of differentiation 4-positive T cells and granulocytes in type 2 AIP or immunoglobulin G4-producing plasma cells in type 1 AIP. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and the cellular immune responses.

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INTRODUCTION

Autoimmune pancreatitis is a recently discovered form

of pancreatitis and represents one of the diseases of the pancreas which can be cured and healed medically[1]. In recent years, several diagnostic criteria have been developed, such as those coming from Japan, South Korea, the United States and Italy[2]. The Japanese criteria are mainly based on radiological appearance while, in addition to imaging, the American and South Korean criteria are based on extra-pancreatic organ involvement and response to steroids, and the Italian diagnostic criteria are based on pathological findings. International consensus diagnostic criteria have subsequently been developed and, although a complete consensus has not yet been achieved, most experts agreed that the clinical phenotypes associated with the histopathologic patterns of lymphoplasmacytic sclerosing pancreatitis (LPSP) [Autoimmune pancreatitis (AIP) without granulocytic epithelial lesions (GELs)] and idiopathic duct-centric pancreatitis (IDCP) (AIP with GELs) should be referred to as type 1 and type 2 AIP, respectively[3]. The main characteristics of the two types of AIP are reported in Table 1. This will allow additional study of and the identification of specific markers of both forms of AIP; at present, the disease associated with IDCP can be definitively diagnosed only by histological examination since steroid trials cannot differentiate diseases associated with LPSP from those associated with IDCP. Type 1 AIP predominantly in Japan[4-6] whereas type 2 AIP was proposed and developed predominantly in Europe on the basis of its histopathological features[7]. Most importantly, in type 1 AIP the pancreatic manifestation is associated with other extrapancreatic disorders resembling an immunoglobulin G4 (IgG4)-related disease (IgG4-RD)[8,9]. We reviewed the current concepts regarding the pathophysiology of this intriguing disease, focusing on the importance of the humoral and the cellular immune responses.

PATHOGENESIS

Although both subtypes undergo remission when treated with corticosteroids[10,11], there is little agreement regarding their pathogenesis. The categorization of AIP as an autoimmune disorder is based on the observation that the disease is associated with the infiltration of immune cells into pancreatic tissue, and that the disease dramatically responds to steroid therapy. The pancreas of a patient with AIP is often infiltrated by various types of immune cells, including cluster of differentiation (CD) 4-positive T cells and granulocytes in type 2 AIP or IgG4-producing plasma cells and B-lymphocyte antigen CD20 in type 1 AIP[12].

SERUM IMMUNOLOGICAL FEATURES

Even if high circulating serum IgG4 levels have been proposed as a marker of AIP with good accuracy in differentiating between AIP and the overall controls, pancreatic cancer and other autoimmune diseases[13], other substances have also been reported in AIP. Hyper-

gammaglobulinemia has been reported with a frequency ranging from 37% to 76%[1]. Levels of autoimmune antibodies, including antinuclear antibody, anticarboanhydrase 2, antismooth muscle antibody, antihuman lactoferrin and rheumatoid factor and pancreatic secretory trypsin inhibitor may all be elevated in a varying proportion of patients[1]. Higher concentrations of circulating leptin[14] as well as high levels of peptide AIP_{1-7} which showed homology with an amino acid sequence of the plasminogen-binding protein of *Helicobacter pylori* and with ubiquitin-protein ligase E3 component n-recognin 2, an enzyme highly expressed in acinar cells of the pancreas have been also reported[15]. However, it is unclear whether the formation of these antibodies constitutes a pathogenetic event or whether they represent an associated epiphenomenon of AIP[16-18]. From a practical point of view, only IgG4 seems to be an interesting molecule for understanding the pathophysiology of type 1 AIP whereas altered cellular immune response is an interesting tool for understanding the pathophysiology of type 2 AIP.

IGG4: ITS PROPERTIES AND ROLE IN AIP

Human IgG subclasses are numbered according to their concentration in plasma; thus, IgG1 is the most abundant (greater than 50% of total IgG) while the amount of IgG4 is scarce, usually less than 5%. A polyclonal antiserum to one Ig class does not cross-react with other Ig classes, but antibodies to an Ig subclass will usually cross-react extensively with other subclasses of the same class. For the most part, IgG antibodies to bacterial polysaccharides belong to the human IgG2 subclass and a similar association was discovered between IgG antibodies to allergens and the IgG4 subclass[19,20].

It has been demonstrated that IgG4 antibodies are non-precipitating and behave like monovalent antibodies[21]. An unusual feature of IgG4 may explain its monovalency[22]; upon eelectrophoretic analysis, a substantial part of IgG4 was found to lack interchain disulphide bonds and, thus, to be a half-molecule of one heavy chain plus one light chain. This phenomenon was shown to be due to a single amino acid located at the site of the bond which differs between IgG1 and IgG4; a proline in IgG1 is replaced by a serine in IgG4. Mutating this serine into proline abolished the appearance of half-molecules on sodium dodecyl sulfate electrophoresis[23]. However, no half-molecules were found upon size exclusion chromatography, and this exchange process seems to be irrelevant *in vivo*.

There is also a peculiar characteristic of IgG4, namely its tendency to interact with other immunoglobulins. This has been studied in relation to the IgG rheumatoid factor[24]. IgG4 was found to possess an intrinsic affinity for IgG coated to a solid phase. This binding activity was not located in its variable domains, but in its constant domain. However, using labeled IgG4, it can be shown that IgG4 will also bind to coated IgG4. To further com-

	Type 1 AIP	Type 2 AIP
Table 1 Epidemiological, laboratory, pathological and clinical characteristics of type 1 and type 2 autoimmune pancreatitis		
Age	Adult	Child and adult
Gender	Usually male	Equal
Serum IgG4 levels	Elevated	Normal
Histology	Lymphoplasmacytic sclerosing pancreatitis	Idiopathic duct-centric pancreatitis
IgG4 plasma cells	Well represented	Rare
Granulocytic epithelial lesions	Absent	Present
Relapse rate	High	Low
Extra-pancreatic lesions	IgG4-related disease: hypophysitis, pachymeningitis, perineural mass, chronic sclerosing dacryoadenitis, chronic sclerosing sialadenitis, lymphadenopathy, thyroiditis or hypothyroidism, pseudolymphoma, breast inflammatory pseudotumor or mastitis, pulmonary inflammatory pseudotumor, nodular pleuritis, chronic gastritis, Vater's ampulla pseudotumor, sclerosing cholangitis, lymphoplasmacytic sclerosing cholecystitis, hepatic inflammatory pseudotumor, autoimmune hepatitis, retroperitoneal fibrosis, periaortitis/periarteritis, inflammatory aneurysm, tubulointerstitial nephritis	Inflammatory bowel disease

AIP: Autoimmune pancreatitis; IgG4: Immunoglobulin G4.

plicate the situation, IgG4 with irrelevant specificity was found to bind to IgG4 antibody bound to its antigen[25]; this is a potential source of artifacts in analytical assays, not only for the measurement of bi-specific IgG4, but also for the measurement of the IgG4 antibody in general. In the case of the measurement of the bispecificity of IgG4, this "non-specific" binding was blocked by adding pooled immunoglobulins to the incubation buffer.

Total IgG4 levels are low in infancy and, thereafter, tend to increase; this presumably reflects a dependency on the maturity of accessory cells (macrophages, dendritic cells, *etc.*) which are important producers of interleukin (IL)-10. Moreover, some of the IL-10 effects are mediated via such accessory cells[26]. In fact, it has been suggested that AIP patients may be exposed to high doses of unknown disease-specific antigens, resulting in the activation of both Th1-type immune cells and regulatory T cells *via* IL-10[27].

The slow kinetics of IgG4-expressing cells is also reflected in IgG4-specific antibody levels. The IgG4/IgG1 ratio of antibodies to common foods is lower in infancy than in adolescence. This shift in the IgG4/IgG1 antibody may be related to the chronic stimulation requirement for IgG4 production, as previously discussed. This shift to IgG4 is, however, only partially due to an earlier appearance of IgG1 antibodies; it also reflects an earlier decline of IgG1 antibodies[28].

The requirements for the class switch to IgG4 are similar to those for IgE because both depend on IL-4/IL-13 induction[29-32]. Both are therefore considered to be part of the Th2 immune response. In relation to allergen-specific immunotherapy, it is sometimes suggested that a switch occurs from IgE production to IgG4 production. While a B cell can switch sequentially, such a sequential switch can transform an IgG4-producing B cell into an IgE-producing B cell, but not the other way around as a consequence of the sequence order in which the genes for the isotypes are arranged on the chromosome[30-32].

One of the effects of this common dependency on Th2 cells is that antigens which induce IgE responses are also good inducers of IgG4 responses. There are probably some regulatory differences before the class switch because the occurrence of IgG4 antibodies without IgE antibodies is not uncommon. One type of regulation is particularly important: the effects of IL-10 and related cytokines. Interleukin-10 interferes with the class switch[26] which affects both IgE and IgG4 production[33]. In addition, IL-10 is presumably needed to drive the differentiation of IgG4-switched B cells to IgG4-secreting plasma cells[34]. In addition to IL-10, IL-21 has also been found to increase IgG4 production *in vitro*[35,36]. Increased IL-21 production is characteristic of certain autoimmune diseases and is likely to contribute to autoantibody production as well as to the pathologic features of autoimmune disease[37]. In contrast, IL-21 may function as a co-adjuvant to enhance antibody responses and thereby facilitate host defense to malignances and infectious diseases[37]. Thus, the critical role of IL-21 in promoting humoral immune responses makes it an important focus of potential therapeutic interventions under conditions characterized by either the overproduction of pathogenic autoantibodies or the underproduction of protective antibodies.

The "modified Th2 response" was first used in relation to the antibody response to cat allergen[38], and it refers to subjects with IgG4 antibodies without demonstrable IgE antibodies. As the presence of IgG4 antibodies indicates a Th2 response, the absence of IgE antibodies is unexpected. However, this situation is quite common and it is seen in most beekeepers and in individuals having occupational exposure to protein antigens, such as rodent allergens in the animal house and/or exposure to mammalian serum albumin in the animal blood processing industry[39], and usually produces this phenotype[40,41]. Therefore, the modified Th2 response seems to be the typical response to an innocuous antigen[42,43]. It is intriguing that this type of response is not found in all situations where allergen exposure does not

result in IgE production. This is true not only for IgG4, but also for IgG1. The presence of high-affinity IgG antibodies (IgG1 and/or IgG4) to pollen- or mite allergens is much more common in subjects with allergen-specific IgE than in IgE-negative subjects; this difference is more marked for some allergens than for others which suggests that not all allergens are equal. However, some allergens do not induce an IgG antibody response at all whereas others induce an IgG (IgG1 and/or IgG4) response without IgE[44].

An important aspect of the IgG4 response is the slow manifestation of IgG4 antibodies. It usually takes many months of repeated antigen exposure before IgG4 responses become prominent. This is well known in the sequential analysis of sera from novice bee-keepers[45], and the analysis of sequential samples from patients who received subcutaneous allergen-specific immunotherapy shows the same pattern. It is likely that the production of sufficient IL-10 is a rate-limiting step.

CELLULAR IMMUNE ACTIVATION

CD4 or CD8 T lymphocytes and IgG4-bearing plasma cells have been found in the pancreatic parenchyma and other involved organs in AIP[46-49]. It seems that factors regulating T-cell function influence the development of AIP. The cytotoxic T-lymphocyte antigen 4 gene is an inhibitory receptor expressed on the cell surface of activated memory T cells and on $CD4^+$ $CD25^+$ regulatory T cells, and acts largely as a negative regulator of T-cell responses[50]. CTLA4 may modulate positive T-cell costimulatory signals by competing with the CD28 molecule for engagement with the B7 molecules CD80 and CD86 localized on antigen-presenting cells. In addition, and *CTLA4 + 49A/G* single nucleotide polymorphisms (SNPs) have been associated with susceptibility to autoimmune diseases, such as type 1 diabetes, autoimmune thyroid disease, autoimmune hepatitis, and primary biliary cirrhosis[50]. Another form of *CTLA4*, secreted by resting T cells, can suppress T-cell activation and this soluble isoform of *CTLA4* (s*CTLA4*) is present in human serum, and it is elevated in patients with autoimmune diseases, such as autoimmune thyroid disease[51], systemic lupus erythematosus[52], and myasthenia gravis[53]. The + 6230G/A SNP in the 3' untranslated region of *CTLA4* has been also found in Graves' disease, type 1 diabetes[54] and Umemura *et al*[55] have demonstrated that AIP is closely associated with the *CTLA4 + 6230* SNP and serum sCTLA4 levels and that *CTLA4* gene plays an important role in the pathogenesis of AIP.

It has also been reported that *DRB1*0405* and *DQB1*0401* mutations are significantly more frequent in patients with AIP when compared to those with chronic calcifying pancreatitis[56], even if these initial and promising findings were not confirmed by two recent studies[57,58]. Furthermore, Park *et al*[57] found that only *DQB1*0302* had a significant association with the relapse of AIP. Finally, it has been found that the polymorphic genes (CTLA-4 49A polymorphism and -318C/+ 49A/

CT60G haplotype) encoding cytotoxic T lymphocyte-associated antigen 4, a key negative regulator of the T-cell immune response, are associated with AIP in a Chinese population[59]. Even if these data are not concordant, it is possible that physiological IgG4 responses are induced by prolonged antigen exposure and controlled by type 2 helper T cells[18]. A possible explanation may come from genetically-modified animals which were produced to mimic AIP.

ANIMAL MODELS FOR STUDYING THE PATHOPHYSIOLOGY OF AIP

We believe that due the no high incidence of AIP, the animal models are important in helping the researchers to test new pathogenetic hypotheses on AIP and also new drugs able to treat this disease.

It has been demonstrated that MRL/Mp mice develop a form of autoimmune pancreatitis and that the administration of polyinosinic: polycytidylic acid may substantially shorten the time course and increase the frequency of both pancreatitis and biliary involvement[60]. The experimental model for inducing inflammatory bowel disease, *i.e.*, IL-10$^{-/-}$ mice, has been also used for developing type I AIP[61]. It is also possible induce AIP by immunization with lactoferrin, carbonic anhydrase or other antigens, or by alterations to the intestinal flora[62,63]. When applied, these models may answer the question regarding the first events which may lead to AIP, and to test novel therapeutic modalities, especially those regarding cellular immune activation.

CONCLUSION

Several questions remain open in the pathophysiology of AIP. The interrelationship with allergies and the multivisceral involvement in patients with AIP should be better evaluated in order to answer the question of whether IgG4 disease is initiated by allergens. In this respect, we would point out that we have recently reported an association between anisakis infection and AIP[64], and worms are well-known inducers of allergic phenomena [31]. Finally, we also need to investigate whether type 1 or type 2 AIP are different diseases or different presentation of the same illness.

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2014 ADVANCES IN PANCREATITIS

Review of the diagnosis, classification and management of autoimmune pancreatitis

Derek A O'Reilly, Deep J Malde, Trish Duncan, Madhu Rao, Rafik Filobbos

Derek A O'Reilly, Deep J Malde, Trish Duncan, Department of Hepatobiliary and Pancreatic Surgery, North Manchester General Hospital, Manchester M8 5RB, United Kingdom

Derek A O'Reilly, Institute of Cancer Sciences, University of Manchester, Manchester Academic Health Science Centre, Manchester M8 5RB, United Kingdom

Madhu Rao, Department of Histopathology, North Manchester General Hospital, Manchester M8 5RB, United Kingdom

Rafik Filobbos, Department of Radiology, North Manchester General Hospital, Manchester M8 5RB, United Kingdom

Author contributions: O'Reilly DA, Malde DJ and Duncan T wrote the clinical sections; Rao M contributed to the pathological section; Filobbos R supplied the radiology section and images.

Correspondence to: Derek A O'Reilly, PhD, FRCS, Consultant HPB Surgeon and Honorary Senior Lecturer, Department of Hepatobiliary and Pancreatic Surgery, North Manchester General Hospital, Manchester M8 5RB, United Kingdom. doreilly@doctors.org.uk

Telephone: +44-161-7202277 Fax: +44-161-7202228

Abstract

Autoimmune pancreatitis (AIP) is a rare form of chronic pancreatitis, with as yet undetermined incidence and prevalence in the general population. Our understanding of it continues to evolve. In the last few years, 2 separate subtypes have been identified: type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. International criteria for the diagnosis of AIP have been defined: the HISORt criteria from the Mayo clinic, the Japan consensus criteria and, most recently, the international association of pancreatology "International Consensus Diagnostic Criteria". Despite this, in clinical practice it can still be very difficult to confirm the diagnosis and differentiate AIP from a pancreatic cancer. There are no large studies into the long-term prognosis and management of relapses of AIP, and there is even less information at present regarding the Type 2 AIP subtype. Further studies are necessary to clarify the pathogenesis, treatment and long-term outcomes of this disease. Critically for clinicians, making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance and the greatest challenge.

Core tip: Type 1 autoimmune pancreatitis (AIP) is the pancreatic manifestation of a multiorgan disease, named immunoglobulin G4 (IgG4)-related disease while type 2 AIP is a pancreas specific disorder not associated with IgG4. Making the correct diagnosis and differentiating the disease from pancreatic cancer is of the utmost importance; an agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient.

INTRODUCTION

As early as 1961, Sarles *et al*[1] described a form of idiopathic chronic pancreatitis with obstructive jaundice and hypergammaglobulinaemia, with the suspicion that there

was an underlying autoimmune process. It was not until 1995, when Yoshida *et al*[2] coined the term "autoimmune pancreatitis" (AIP) that this concept was widely accepted and AIP differentiated from other forms of chronic pancreatitis. Since then, progress has been made in our understanding of the pathophysiology of AIP; type 1 AIP has been recognised as the pancreatic manifestation of a multiorgan disease, named IgG4-related disease, while type 2 AIP is a pancreas specific disorder not associated with IgG4[3,4]. This review gives an overview of current thinking on the pathology of AIP, its clinical features (including serology), classification and treatment. Emphasis is placed upon the diagnostic challenge of distinguishing AIP from pancreatic cancer.

SEARCH STRATEGY

This review of the English language literature on the classification, diagnosis and management of autoimmune pancreatitis is based on papers contained within the PubMed database. Individual searches of the PubMed database were performed with the boolean operator AND, using the terms: "Autoimmune pancreatitis", "Acute pancreatitis", "Chronic pancreatitis", "Pancreatic cancer". The abstracts were screened for eligibility and all relevant publications were requested as full-text articles. References used in requested papers were then checked for any further studies of potential interest.

PATHOPHYSIOLOGY OF AIP

A definitive autoantigen for AIP has not yet been identified. Human leucocyte antigen (HLA) association studies in Japan have reported an association with HLA serotypes DRB1*0405 and DQB1*0401[5]. This was not confirmed in a Korean study but DQβ1-57 without aspartic acid was associated with disease relapse[6]. Single nucleotide polymorphisms identified in association with either disease susceptibility or recurrence include: cytotoxic T-lymphocyte associated antigen 4, tumour necrosis factor-α and Fc receptor-like 3[7]. However, studies of genetic risk factors in AIP remain at an early stage of investigation. A genome-wide association study in AIP would likely advance our understanding significantly.

Potential initiating mechanisms include bacterial infection and molecular mimicry[7]. Substantial homology exists between human carbonic anhydrase Ⅱ and the α-carbonic anhydrase of Helicobacter pylori[8]. In theory, antibodies directed against bacterial components could behave as autoantibodies by means of molecular mimicry in genetically predisposed persons[7]. Thus, autoimmunity is widely regarded as the initial stimulus for the Th2-cell immune response associated with AIP. Antibodies directed against potential autoantigens, such as carbonic anhydrase, lactoferrin, trypsinogen and pancreatic secretory trypsin inhibitor, may give rise to the systemic manifestations of AIP[7-11].

Studies using animal models of experimental autoimmune pancreatitis have significant limitations, as the disease does not occur spontaneously. Current models exhibit considerable variation in target antigens, differing methods for immune staining and differing mouse strains but have provided evidence that the disease is most likely T cell mediated, with highly beneficial effects observed with agents such as the mammalian target of rapamycin (mTOR) inhibitor, sirolimus, which increases the number and activity of regulatory T-cells[4].

SUBTYPES OF AUTOIMMUNE PANCREATITIS

Type 1

This is the more classically described and recognised form of the disease. It is now recognised as a pancreatic manifestation of an immunoglobulin G4 (IgG4) related systemic disease[4,7,12-14]. It is associated with histological findings of a lymphoplasmacytic sclerosing pancreatitis (LPSP). This consists of a dense lymphoplasmacytic infiltration and fibrosis involving the pancreatic lobules, ducts and peripancreatic adipose tissue. Storiform or "swirling" fibrosis and obliterative phlebitis are also characteristic features[15-17]. The lymphoplasmacytic infiltrate is also rich in IgG4 positive cells[18]. It is frequently associated with sclerosing extrapancreatic lesions such as sclerosing cholangitis, retroperitoneal fibrosis and sclerosing sialadenitis[13,19-21]. Type 1 AIP tends to affect older males, with 80% of patients being over 50 years of age at the time of presentation. It is also associated with elevation in serum levels of IgG4 in up to 75% of patients[19,20].

The HISORt criteria from the Mayo clinic[22] and the Japanese consensus criteria[23] were mainly produced to facilitate the diagnosis of Type Ⅰ AIP.

Type 2

This is a relatively recently described form of AIP[3,4]. It has a unique histological pattern, consisting of an idiopathic duct-centric pancreatitis or AIP with a granulocytic epithelial lesion. The inflammation is centred on the exocrine pancreatic system, with neutrophilic infiltration within the lumen and epithelium of the interlobular ducts being a characteristic feature. The neutrophils are sometimes so numerous that microabscesses can be seen in the lobules and ducts. The entire wall of the duct may be infiltrated by neutrophils and plasma cells. The infiltrate frequently involves the duct epithelium and can obliterate it. It differs from LPSP in that there is little obliterative phlebitis and the inflammatory infiltrates have few IgG4 positive cells[24,25].

Much less is known regarding the clinical features of Type 2 AIP. However it appears to be associated with a younger subset of patients and there is no gender preponderance. There also appears to be an association with ulcerative colitis. Type 2 AIP patients usually have a dramatic response to steroid therapy, associated with a low frequency of relapse[25]. Until recently, existing criteria have not been that helpful in the diagnosis of type 2 AIP, but with recent publication of the International
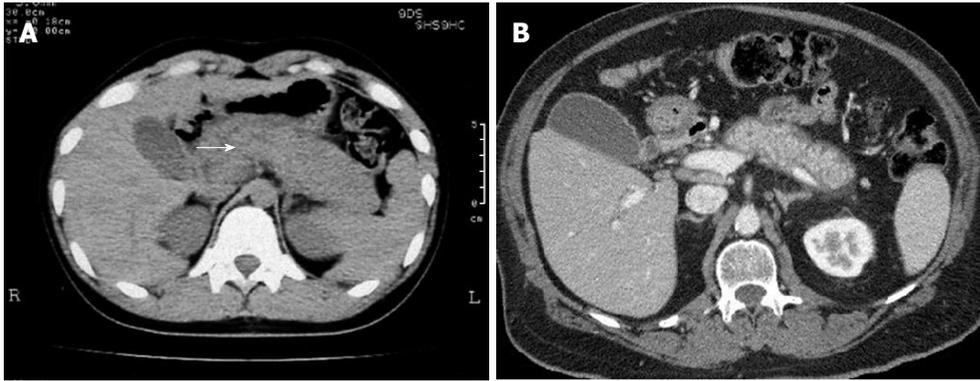
Figure 1 Computed tomography. A: Computed tomography (CT) findings in autoimmune pancreatitis: Showing diffuse enlargement and a "sausage like" appearance of the pancreas (arrow); B: Axial contrast enhanced CT image demonstrating a characteristic low signal rim or halo surrounding the body and tail of the pancreas in another patient with autoimmune pancreatitis.

Association of Pancreatology (IAP) diagnostic guidelines[26], it is anticipated that more data will confirm and further characterise this subtype.

Variation in the geographic distribution of the two subtypes may help to explain the heterogeneity of disease morphology observed worldwide.

CLINICAL PRESENTATION

The presentation of AIP is varied, but a classical picture is obstructive jaundice, often painless or with mild epigastric pain. Less commonly, new onset diabetes or symptoms of pancreatic insufficiency and weight loss may occur. A rarer presentation is acute pancreatitis and its sequelae. A characteristic feature of type 1 AIP is extrapancreatic other organ involvement. In Type 1 AIP the majority are male and over the age of 50. Some patients are only diagnosed post-operatively, having had a resection for a presumed pancreatic cancer.

The clinical picture in Type 2 autoimmune pancreatitis appears to affect a younger cohort of patients, more likely in their 4th decade of life and there is no gender preponderance. There are more reports of this group presenting with acute pancreatitis, and a higher frequency of association with ulcerative colitis[25]. However, the numbers of patients reported in the worldwide literature are still very small and further clarity is expected to emerge with time, to further define this subgroup.

SEROLOGY

Type 1 AIP is associated with a number of serological abnormalities, in particular an elevated IgG4[18,19]. Hamano *et al*[19] reported that a cut-off value of 135 mg/dL for serum IgG4 concentration differentiates AIP from pancreatic cancer with an accuracy of 97%, a sensitivity of 95% and specificity of 97%. An elevated IgG4 is however not diagnostic of Type 1 AIP, but is a characteristic along with other identified criteria. The Mayo clinic reported a sensitivity, specificity and positive predictive value of 76%, 93% and 36% respectively, using a cut-off value for IgG4 of 140 mg/dL[27]. Elevated IgG4 levels

also may be found in PSC, acute and chronic pancreatitis and up to 10% of patients with pancreatic cancer[19]. Serum IgG4 of more than 2 times the upper limit of normal greatly increases the specificity for AIP.

Other elevated markers may include: rheumatoid factor, carbonic anhydrase, antilactoferrin and antinuclear antibodies[9,10]. A study from Frulloni *et al*[28] in Italy identified an anti plasminogen-binding peptide antibody which was elevated in 94% of their AIP patients. In this cohort of AIP patients, they had a relatively low prevalence of elevated IgG4 (at only 54%). This was a single centre study of 20 patients and clearly more studies are needed to assess this and other autoantibodies as potential markers for AIP and as aids to distinguish AIP from pancreatic malignancy.

IMAGING

Imaging is essential in establishing a diagnosis of AIP. Three different forms of the disease process can be seen, including diffuse, focal or multifocal disease, with the diffuse form being the most common. A contrast enhanced computed tomography (CT) scan is the gold standard for investigation as it is essential to look for a pancreatic malignancy and evidence of metastatic disease. Figure 1A shows the contrast enhanced CT findings characteristic of Type 1 AIP: a diffusely enlarged or "sausage shaped" pancreas with loss of the normal pancreatic clefts and delayed and peripheral rim enhancement[29]. Figure 1B shows a characteristic surrounding hypoattenuating/low signal rim or halo on CT. Generally there is minimal associated peripancreatic soft tissue stranding and rarely inflammation of the mesentery. Local peripancreatic lymphadenopathy can be observed. Pancreatic calcification and pseudocyst formation is not a recognised typical finding in autoimmune pancreatitis. CT may also find extra pancreatic lesions such as retroperitoneal fibrosis.

The focal form of the disease is less common and is characterized by a focal mass lesion within the pancreas and can be mistaken for pancreatic malignancy (Figure 2). Normally dilatation of the pancreatic duct is less marked in autoimmune pancreatitis than that associated
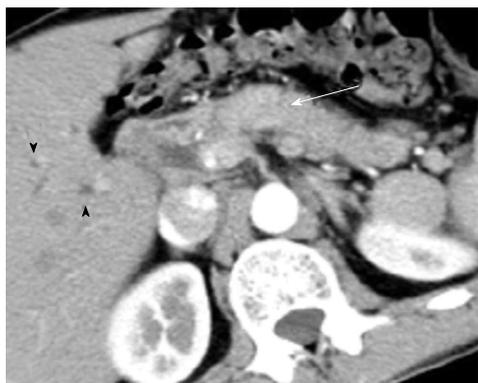
Figure 2 Focal enlargement of the pancreatic parenchyma in the head of the pancreas (arrow), and dilatation of the intrahepatic bile ducts visible (arrowheads).

Figure 3 Endoscopic retrograde cholangiopancreatography findings of multiple and focal strictures and dilatation in the intrahepatic bile ducts in autoimmune pancreatitis.

with pancreatic malignancy. Typically the main pancreatic duct is irregularly narrowed in affected segments of the pancreas. In the multifocal form of the disease, the pancreatic duct is of normal calibre in non affected segments. Magnetic resonance imaging (MRI) shows diffuse or localised enlargement of the pancreas with lower density in T1 weighted images and higher density in T2 weighted images compared with each of the liver images.

Sclerosing cholangitis is observed in a proportion of patients with autoimmune pancreatitis and can be seen in isolation. The intrapancreatic portion of the common bile duct is the most affected segment of the biliary tree. Affected segments of the biliary tree demonstrate irregular stricturing and associated contrast enhancement. Generally strictures associated with autoimmune disease are long and continuous whereas multifocal short strictures are more typical of primary sclerosing cholangitis (PSC), although differentiation between the two can be difficult in some cases (Figure 3).

Endoscopic ultrasound (EUS) is being used more frequently for pancreatic core biopsies, which acts as an aide to histological diagnosis and is likely superior to fine needle aspiration (FNA)[30]. Typical EUS findings in AIP include: diffuse hypoechoic spots, absence of a discreet mass and chronic inflammatory cells on aspiration cytology. Mizuno *et al*[30] and Levy *et al*[31] have demonstrated the benefits of the use of EUS-guided biopsies to aid in the diagnosis of AIP[32]. Future refinement of diagnosis may be obtained with the use of contrast-enhanced EUS and elastography[4]. The use of positron emission tomography (PET) and its potential role for diagnosis of AIP is yet to be validated[33].

OTHER ORGAN INVOLVEMENT

In Type 1 AIP, which may be considered part of an IgG4 systemic disease process, there are a significant number of associated extrapancreatic lesions. The most common are: hilar lymphadenopathy, sclerosing cholangitis, retroperitoneal fibrosis, salivary and lacrimal gland involvement and tubulointerstitial nephritis[21,22,34-37]. There are other conditions that have been less frequently reported,

such as hypophysitis and chronic thyroiditis. It is this link to other organ involvement that led clinicians to consider AIP as part of a systemic IgG4 related disease, analogous to sarcoidosis, another systemic disease in which diverse organ manifestations are linked by the same histopathological characteristics[7].

Biliary disease is one of the most common extrapancreatic manifestations of AIP. Although the main cause of jaundice in AIP is obstruction at the level of the intrapancreatic portion of the common bile duct, associated with an inflammatory pancreatic head mass, stricturing in the rest of the biliary tree is increasingly recognised. This condition has been termed IgG4-associated cholangitis (IAC) and has been reported to occur in 20%-88% of cases of AIP[38]. A possible overlap between IAC and PSC is also suggested by the finding that 9%-36% of patients with PSC have increased serum IgG4 levels, compared with less than 1% in other liver diseases[39,40]. Of note, PSC patients with raised serum IgG4 levels have a more rapid progression to liver transplantation compared to those with normal levels[38].

Extrapancreatic disease can be a useful factor in the diagnosis of autoimmune pancreatitis, distinguishing it from pancreatic cancer, and forms part of the HISORt criteria. It also provides collateral evidence for AIP, according to the IAP diagnostic guidelines. The evidence to support the association between these conditions and AIP include: multiple reports indicating frequent or intimate concurrence, extrapancreatic pathological findings of severe lymphoplastic infiltration and storiform fibrosis with numerous IgG4 positive plasma cell infiltrations and a combined favourable response to steroid therapy[23,26,41].

DIAGNOSIS OF AIP

There is no single diagnostic test for AIP and there is significant variation in clinical practice worldwide, particularly between Asia and North America/Europe. The biggest challenge associated with the diagnosis of AIP is that it can closely resemble pancreatic cancer. Most commonly AIP presents with obstructive jaundice and pancreatic enlargement; other worrying symptoms such as

Table 1 The Mayo clinic HISORt criteria for the diagnosis of autoimmune pancreatitis

Category	Criteria
Histology	One of the following:
	Periductal lymphoplasmacytic infiltrate with obliterative phlebitis and storiform fibrosis (LPSP)
	Lymphoplasmacytic infiltrate with storiform fibrosis showing abundant IgG4 positive cells (> 10 cells/HPF)
Imaging (CT)/(MRI)	Typical; diffusely enlarged gland with diffuse rim enhancement, diffusely irregular attenuated pancreatic duct
	Other; focal pancreatic mass or enlargement; focal pancreatic duct stricture; pancreatic duct stricture, pancreatic atrophy; pancreatic calcification or pancreatitis
Serology	Elevated serum IgG4 level
Other organ involvement	Hilar/intrahepatic biliary strictures, persistent distal biliary strictures, parotid or lacrimal gland involvement, mediastinal lymphadenopathy or retroperitoneal fibrosis
Response to steroid therapy	Resolution/Marked improvement of pancreatic or extrapancreatic manifestion with steroid therapy

LPSP: Lymphoplasmacytic sclerosing pancreatitis; CT: Computed tomography; MRI: Magnetic resonance imaging; IgG4: Immunoglobulin G4; HPF: High powered field.

weight loss and new onset diabetes may also be present. Less commonly AIP can present with features of acute pancreatitis or unexplained pancreatic insufficiency. Misdiagnosis at this stage has the potential to be catastrophic, as an undiagnosed cancer may cause delay or loss of the opportunity for potential curative cancer surgery. The opposite scenario of a pancreatoduodenectomy being undertaken for benign disease (with its high risk of morbidity and mortality) is also unsatisfactory.

In 2002 the Japan Pancreas Society published guidelines for diagnosis of AIP. These were updated in 2006 and again in 2009. The HISORt criteria from the Mayo[22] clinic require histology, imaging, serology, other organ involvement and response to therapy for diagnosis. The inclusion of response to steroids as part of the diagnosis is one of the criteria that differentiates the Mayo recommendations from the Japanese. In Japan, endoscopic retrograde pancreatography (ERP) is routinely performed to aid in the diagnosis of AIP. More recently, the IAP has published their International consensus diagnostic criteria (ICDC)[26], in an attempt to bridge the divide in clinical practise around the globe and offers criteria for the diagnosis of both subtypes of AIP.

SUMMARY OF DIAGNOSTIC CRITERIA

Guidelines regarding diagnostic criteria vary worldwide. Although criteria have been developed by other groups, the most influential come from the United States[22], Japan[23] and the International Association of Pancreatology[26]. Below are the definitions from these three different groups.

Japan/Asian

In 2002 the Japan Pancreas society published their data for the diagnosis of AIP; this was further revised in 2006. In 2009 Okazaki *et al*[23] published the Japanese consensus guidelines for management of autoimmune pancreatitis. There are 3 main criteria. For the diagnosis to be confirmed, criterion 1 must be present along with criterion 2 and/or criterion 3.

Imaging: Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or segmental enlargement of the pancreas with imaging studies such as: Ultrasound, CT, MRI or ERP.

Serology: High serum gammaglobulin IgG or IgG4, or the presence of autoantibodies, such as antinuclear antibodies or rheumatoid factor.

Histology: Marked inter-lobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area, occasionally with lymphoid follicles in the pancreas.

There is an optional criterion for patients fulfilling criterion 1 alone: a response to steroid therapy, with the caveat that malignancy of the pancreas or biliary tract must be excluded. In 2006, a mandatory ERP became part of these guidelines.

United States

The Mayo clinic HISORt criteria are based on 5 main diagnostic criteria: histological findings, imaging, serology, other organ involvement and response to steroid therapy[22,42]. The detailed features are listed in Table 1. Essentially, use of these criteria enable patients to be categorised into three diagnostic groups [diagnostic pancreatic histology, typical imaging and serology, steroid responders (after careful work-up to exclude cancer)]. Patients in one or more of these categories are deemed to have AIP.

International association of pancreatology

The goals of the IAP were to develop international consensus on the diagnostic criteria that can be applied worldwide, to safely diagnose AIP and to avoid a misdiagnosis of pancreatic cancer[26]. They reviewed all existing criteria, including the Japanese and HISORt. The consensus opinion was that the terms type 1 and type 2 should be used to describe the clinical profiles associated with LPSP and idiopathic duct-centric pancreatitis, respectively. Tables 2-4 shows the diagnostic criteria for definitive and probable AIP type 1 and 2. This uses a combination of 1 or more of 5 cardinal features of AIP: (1) imaging features of the following: pancreatic parenchyma (on

Table 2 International consensus diagnostic criteria for type 1 autoimmune pancreatitis			
Diagnosis of type 1 AIP			
Diagnosis	**Cardinal feature**	**Imaging evidence**	**Collateral evidence**
Definitive type 1	Histology	Typical/in-derminate	Confirmed LPSP
	Imaging	Typical inderminate	Any level 1/2 ≥ 2 level 1
	Steroid response	Indeterminate	Level 1 S/OOI and Rt OR Level 1 D and level 2 S/OOI/H and Rt
Probable type 1		Indeterminate	Level 2 S/OOI/H and Rt

LPSP: Lymphoplasmacytic sclerosing pancreatitis; AIP: Autoimmune pancreatitis; S: Serology; OOI: Other organ involvement; Rt: Response to steroid therapy; H: Histology

Table 3 International consensus diagnostic criteria for type 2 autoimmune pancreatitis		
Diagnosis of type 2 AIP		
Diagnosis	**Imaging evidence**	**Collateral evidence**
Definitive type 2	Typical/indetermi-nate	Histologically confirmed or clinical inflammatory bowel disease and level 2H and Rt
Probable type 2	Typical/indetermi-nate	Level 2 H/clinical inflammatory bowel disease and Rt

AIP: Autoimmune pancreatitis; Rt: Response to steroid therapy; H: Histology.

CT/MRI) and pancreatic duct [ERCP or magnetic resonance cholangiopancreatography (MRCP)]; (2) serology (IgG, IgG4 and antinuclear antibody); (3) other organ involvement (OOI); (4) histopathology of the pancreas; and (5) response to steroid therapy.

Level 1 and level 2 criteria are then specified, according to the strength that specific findings add to the likelihood of diagnosis. For example, a greater than 2-fold elevation of IgG4 is considered a level 1 criteria; a lesser elevation level 2. Further specification is given for pancreatic ductal and parenchymal appearances, histology and response to steroids. Thus, definite and probable type 1 and type 2 AIP can be diagnosed.

In all cases the criteria are geared towards excluding a diagnosis of pancreatic cancer rather than screening for AIP, *i.e.,* they emphasise specificity rather than sensitivity. Only the IAP guidelines include the diagnostic features of Type 2 autoimmune pancreatitis.

DISTINGUISHING AIP FROM PANCREATIC CANCER

In view of its presentation with obstructive jaundice and pancreatic enlargement, AIP often needs to be distinguished from pancreatic cancer. As ERCP features have been reported to have limited sensitivity to diagnose AIP in Western centres, Figure 4 shows a strategy to aid in differentiation, diagnosis and management of AIP versus pancreatic cancer, based upon the experience and algorithm of the Mayo Clinic[22]. When features highly suggestive of either AIP or pancreatic cancer are present (a low-density mass, pancreatic ductal dilatation, pancreatic duct cut off, upstream pancreatic atrophy or liver lesions suggestive of metastases), the diagnostic and management pathway is usually clear. However, in indeterminate cases, further cancer work-up is required in the first instance. In the event of a negative cancer work-up, a pancreatic core biopsy is helpful in categorising patients if a positive diagnosis can be made. Equivocal or inadequate results are more problematic and a trial of steroids or surgery should be considered.

Using the Mayo Clinic strategy, AIP was successfully distinguished from pancreatic cancer in most patients but 27% required a pancreatic core biopsy, steroid trial or surgery to clarify the diagnosis[43]. Kamisawa *et al*[44] have reported their Japanese strategy when investigating patients presenting with mass lesions. Strategies based upon the Japanese criteria can be simpler but rely on ERP. Despite this, surgery was still required to make a diagnosis in 6 of 37 (16%) patients. Further evaluation and comparison is required to determine the optimal and least invasive diagnostic pathway.

In our view, when distinguishing AIP from pancreatic cancer, the most important tips or principals of diagnosis include the following: (1) clinical presentations not suggestive of AIP include marked cachexia, anorexia and severe pain requiring opiates; (2) a thorough negative work up for other aetiologies should be undertaken, in particular for pancreatic or biliary cancer; (3) histological diagnosis of AIP requires preservation of tissue architecture (showing lymphoplasmacytic infiltrate with >10 IgG4 positive cells/high power field), which renders FNA less helpful for diagnosis; (4) steroid therapy should only be commenced when other aetiologies for pancreatic disease have been excluded, and only in those patients whose response may be adequately assessed. It should not be used as a substitute for a thorough search for the aetiology; (5) objective improvement in the appearance of the pancreas on cross-sectional imaging should be evident within 2 wk of steroid use. Subjective improvement in symptoms or even a decline in serum IgG4 levels can occur in pancreatic cancer or lymphoma and should not be used as response criteria; (6) in AIP, CA 19-9 levels drop with treatment; a rising CA 19-9 suggests this diagnosis is incorrect; and (7) the diagnosis of AIP is difficult. An agreed diagnostic pathway should be in place and a multidisciplinary approach taken with each patient, to ensure that pancreatic cancer patients are not treated with steroids and, conversely, AIP patients not treated with cancer surgery.

INITIAL TREATMENT, MAINTENANCE AND RELAPSE

Although it is well established that spontaneous resolution can occur in up to 30% of cases of AIP[45], symptomatic patients are best treated with corticosteroids (*i.e.,*

Table 4 International consensus diagnostic criteria level 1 and 2 criteria for type 1 and 2 autoimmune pancreatitis

Criterion	Level 1	Level 2
Type 1 AIP		
Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement	Indeterminate: Focal enlargement with delayed enhancement
Ductal imaging (ERP)	Long or multiple strictures (> 1/3 duct length) without upstream dilatation	Focal narrowing without upstream dilatation (< 5 mm)
Serology	IgG4 > 2x upper limit	IgG4 1-2x upper limit
Other organ involvement	Extrapancreatic organ histology. Any 3 of : 1 Lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration 2 Storiform fibrosis 3 Obliterative phlebitis 4 > 10 cells/HPF IgG4-positive cells Typical radiology. Any one of: 1 Segmental/multiple proximal or distal biliary stricture 2 Retroperitoneal fibrosis	Extrapancreatic organ histology including bile duct biopsies. Both of: 1 Marked lymphoplasmacytic infiltration without granulocytic infiltration 2 10 cells/HPF IgG4-positive cells Physical or radiological evidence of at least one of: 1 Enlarged salivary/lachrymal glands 2 Renal involvement
Histology of pancreas	LPSP and 3 of: 1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration 2 Obliterative phlebitis 3 Storiform fibrosis 4 > 10 cells/HPF IgG4-positive cells	LPSP and 2 of: 1 Periductal lymphoplasmacytic infiltrate without granulocytic infiltration 2 Obliterative phlebitis 3 Storiform fibrosis 4 > 10 cells/HPF IgG4-positive cells
Response to steroid (Rt)	Rapid (< 2 wk) radiological demonstration of marked improvement in pancreatic/extrapancreatic manifestations	
Type 2 AIP		
Parenchymal imaging	Typical: Diffuse enlargement with delayed enhancement	Indeterminate: Focal enlargement with delayed enhancement
Ductal Imaging (ERCP)	Long (> 1/3 duct length) or multiple strictures without upstream dilatation	Focal narrowing without marked upstream dilatation (< 5 mm)
Other organ involvement		Clinically diagnosed inflammatory bowel disease
Histology of pancreas	IDCP. Both of: 1 Granulocytic infiltration of duct wall with or without acinar inflammation 2 0-10 cells/HPF IgG4-positive cells	Both of : 1 Granulocytic and lymphoplasmacytic acinar infiltrate 2 0-10 cells/HPF IgG4-positive cells
Response to steroid (Rt)	Rapid (< 2 wk) radiological demonstration of marked improvement in manifestations	

LPSP: Lymphoplasmacytic sclerosing pancreatitis; IDCP: Idiopathic duct-centric pancreatitis; AIP: Autoimmune pancreatitis; IgG4: immunoglobulin G4; ERP: Endoscopic retrograde pancreatography; Rt: Response to steroid therapy; HPF: High powered field.
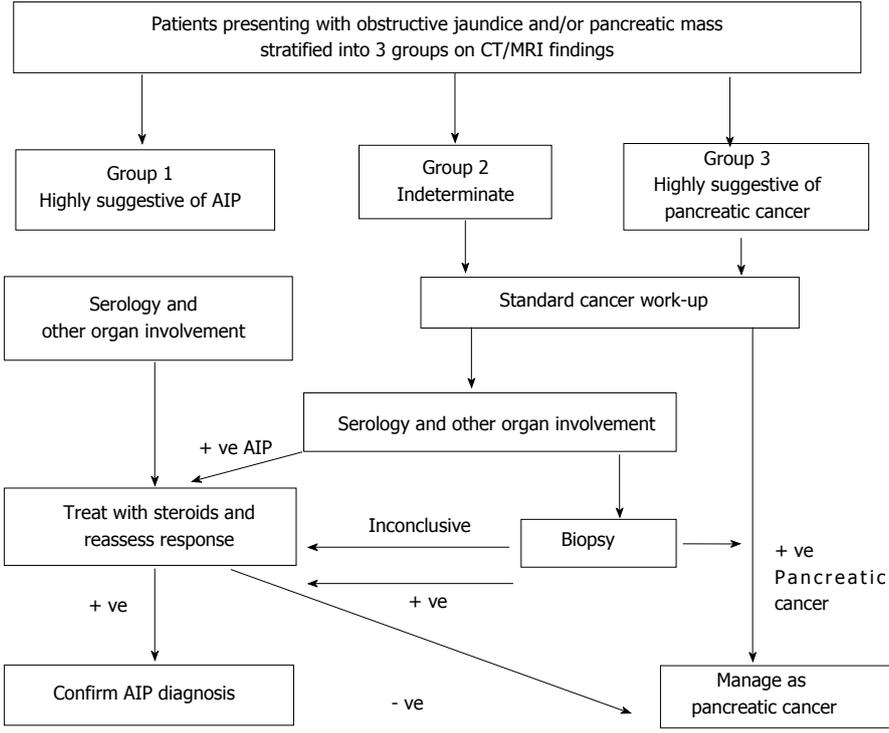

Figure 4 A strategy for distinguishing auto-immune pancreatitis from pancreatic cancer (based upon the Mayo clinic strategy[23]). CT: Computed tomography; MRI: Magnetic resonance imaging; AIP: Autoimmune pancreatitis.
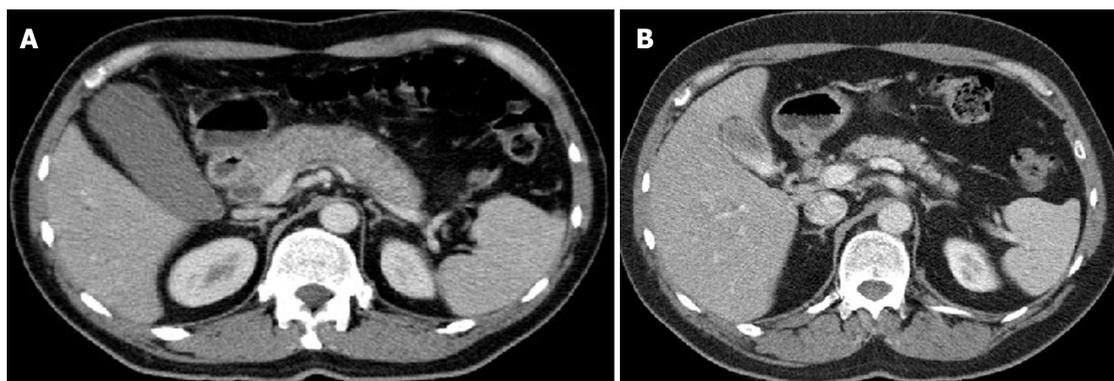
Figure 5 Axial computed tomography image. A: Demonstrating a characteristic sausage shaped enlarged pancreas with surrounding halo in keeping with autoimmune pancreatitis; B: From the same patient 8 mo later following corticosteroid therapy demonstrating response to treatment.

prednisolone). A large multicentre retrospective trial from Kamisawa *et al*[46] in 2009 identified 563 patients with AIP and found that 98% responded to steroid therapy versus 74% that improved without. The response can be dramatic. An improvement of imaging findings, with resolution of pancreatic enlargement and biliary stricturing can be seen following corticosteroid treatment in Figure 5.

Initial steroid dose varies slightly according to guideline. In the Mayo clinic a standard initial dose is 40 mg per day of oral prednisolone, for 4 wk. If there is obvious clinical and radiological improvement, the dose is decreased by 5 mg/wk until it is stopped at 11 wk[47]. The Japanese consensus statement on treatment and prognosis of AIP specifies that an initial oral prednisolone dose for induction of remission of 0.6 mg/kg per day is recommended. The initial dose is administered for 2-4 wk and then gradually tapered. The IAP guidelines specify dose of prednisolone of 0.6-1.0 mg/kg per day with reassessment at 2 wk[26]. The study that formed the basis of the IAP consensus guideline regarding the two week reassessment after a trial of steroid treatment was the prospective study of Moon *et al*[40]. After a 2-wk steroid trial, response to steroids was assessed on the basis of a marked improvement in pancreatic duct narrowing, and a reduction in size of the pancreatic mass. All patients who responded to steroids (15/22) were diagnosed as AIP after a median follow-up of 27 mo, whereas all patients who did not respond to steroids (7/22) were diagnosed with pancreatic cancer, with a complete resection being possible in 6/6 patients who accepted surgery. Induction of remission with rituximab, a monoclonal antibody directed against the CD20 antigen on B lymphocytes, is currently under investigation[4, 48].

Differing rates of tapering are also recommended. Chiefly, the distinction is between the 5 mg/wk reduction of prednisolone, after initial treatment versus a more gradual approach recommended by the Japanese. The Japanese consensus document advocates that the dose be tapered by 5 mg every 1-2 wk, after 2-4 wk at the initial dose, based on changes in the clinical manifestations, biochemical blood tests (such as liver enzymes and IgG or IgG4 levels), and repeated imaging findings (US, CT, MRCP, ERCP). The dose is tapered to a main-

tenance dose over a period of 2-3 mo.

A maintenance dose of 2.5-5.0 mg/d is recommended by the Japanese, to prevent relapse. This is not recommended by the Mayo clinic group, who take the view that the universal use of maintenance therapy is not warranted because the risks of long term steroid use outweighs the benefits[47]. A wide range of relapse rates are reported, from 22%-100%[38]. In the Mayo clinic experience of 78 type 1 AIP patients with a median follow-up of 42 mo, symptomatic disease relapse was seen in 47% patients with a 3-year cumulative relapse rate of 59% in type 1 AIP patients who were medically managed[49]. This wide variation in relapse rates may be due to lack of a uniform definition of disease relapse, short follow-up times, small patient populations, differences in steroid treatment regimens, lack of identification of subtypes and ethnic variation.

Treatment of relapse is effectively achieved with corticosteroids. The Japanese consensus guideline states that remission can be obtained with the same prednisolone dose as the initial dose in most relapsed AIP cases, but that it may be necessary to taper more gradually[50]. In Europe and the United States, azathioprine has often been introduced for the treatment of relapsing disease, despite pancreatitis being a known side-effect of azathioprine. Acute pancreatitis occurs in approximately 2% of cases of azathioprine use, but there is no evidence as yet that this risk is increased in AIP. Some advocate that, as in autoimmune hepatitis (AIH), AIP should be managed by azathioprine, with or without low dose steroids for at least three years. This analogy is not completely convincing; in AIH disease relapse is almost universal in those who cease immunosuppression early whereas the relapse rate is much more variable in AIP. Moreover, in a recent study from the Mayo group, in patients with relapsing AIP, azathioprine was not shown to be superior to another course of steroids alone[51].

Related areas of management include: biliary stenting, treatment of endocrine and exocrine failure and consideration of pancreatic cancer risk in AIP. Patients presenting with obstructive jaundice should certainly be considered for biliary stenting at ERCP. This is the Japanese practice[50] as it fits in with their strategy, which

includes endoscopic pancreatography in an intrinsic role among their diagnostic tests. However, resolution of jaundice occurs in AIP with steroid treatment without stenting, and obviously, this avoids the risks of ERCP. Avoiding the morbidity and mortality associated with ERCP and biliary stenting is also increasingly attempted in suspected pancreatic cancer, as routine preoperative biliary drainage in patients undergoing surgery for cancer of the pancreatic head increases the rate of overall complications[52]. Diabetes mellitus is common in AIP and although improvement has been reported upon commencing steroids, often requires treatment with oral hypoglycemic agents or insulin[47]. Similar considerations apply to exocrine pancreatic failure. Patients should receive pancreatic enzyme supplementation if pancreatic exocrine insufficiency is suspected, based on the presence of clinical features such as: diarrhoea, steatorrhoea, weight loss, metabolic bone disease or vitamin or mineral deficiency. There is no established association between AIP and pancreatic cancer, just case reports of both conditions. It is not unreasonable to suppose the AIP shares a similar association with pancreatic cancer as with other forms of chronic pancreatitis, given the florid inflammatory response that may persist and relapse over years. Careful follow up of these patients will provide the definitive answer to this question but in the interim this seems the prudent approach to take.

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2014 ADVANCES IN PANCREATITIS

Alcoholic pancreatitis: A tale of spirits and bacteria

Alain Vonlaufen, Laurent Spahr, Minoti V Apte, Jean-Louis Frossard

Alain Vonlaufen, Gastroenterology Unit, Hôpital de la Tour, 1217 Meyrin/Genève, Switzerland

Alain Vonlaufen, Laurent Spahr, Jean-Louis Frossard, Department of Gastroenterology, University Hospital, 1211 Geneva, Switzerland

Minoti V Apte, Pancreatic Research Group, South Western Sydney Clinical School, The Ingham Institute, Liverpool, NSW 2170, Australia

Minoti V Apte, Faculty of Medicine, The University of New South Wales, Sydney, NSW 2052, Australia

Author contributions: Vonlaufen A wrote the manuscript and created the figure; Spahr L, Apte MV and Frossard JL critically appraised the manuscript and made additional suggestions.

Correspondence to: Jean-Louis Frossard, MD, Department of Gastroenterology, Geneva University Hospital, 4, rue Gabrielle Perret-Gentil, 1211 Geneva,
Switzerland. jean-louis.frossard@hcuge.ch
Telephone: +41-22-3729340 **Fax:** +41-22-3729366
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Abstract

Alcohol is a major cause of chronic pancreatitis. About 5% of alcoholics will ever suffer from pancreatitis, suggesting that additional co-factors are required to trigger an overt disease. Experimental work has implicated lipopolysaccharide, from gut-derived bacteria, as a potential co-factor of alcoholic pancreatitis. This review discusses the effects of alcohol on the gut flora, the gut barrier, the liver-and the pancreas and proposes potential interventional strategies. A better understanding of the interaction between the gut, the liver and the pancreas may provide valuable insight into the pathophysiology of alcoholic pancreatitis.

Key words: Alcohol; Pancreatitis; Fibrosis; Bacteria; Endotoxin; Lipopolysaccharide

Core tip: There is now clear clinical and experimental evidence that bacteria and bacterial products (such as endotoxin) are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

INTRODUCTION

Chronic alcohol consumption is a known cause of injury to several organs, most commonly the liver and the pancreas, but also to the heart, lungs and brain. However, it is well understood that only a minority of alcoholics will ever develop clinically overt pancreatic or liver damage and even fewer numbers will develop clinically overt disease in both organs simultaneously although subclinical damage to both organs has been reported to coexist[1]. The fact that only some alcoholics appear to be susceptible to clinical pancreatitis or hepatitis has led to a concerted search for additional trigger/initiating factors for alcohol-induced organ damage.

Over the past two decades clinical and experimental studies have demonstrated that endotoxin lipopolysaccharide (LPS), from the bacterial wall of gram negative bacteria of the human gut, plays a central role in the initiation and progression of alcoholic liver disease[2]. This was initially based on clinical observations of elevated plasma endotoxin concentrations in alcoholics with and without liver disease[3,4]. Experimental evidence in support of the association of endotoxin and liver disease in humans was subsequently provided by animal studies demonstrating that alcohol-fed rats challenged with LPS developed hepatic lesions resembling alcoholic hepatitis in humans[5,6].

Conversely, targeted disruption of the LPS receptor toll like receptor 4 (TLR4) in alcohol-fed animals protected against liver injury[7].

Reports of increased endotoxinemia in pancreatitis emerged a decade later. Several studies have linked the degree of endotoxinemia to the severity and prognosis of acute pancreatitis, regardless of its aetiology[8,9] and the impact of endotoxinemia on multiple organ system failure, in particular pancreatitis-associated lung disease has been corroborated by animal studies[10]. However, it remained elusive whether endotoxinemia was a cause or a consequence of pancreatitis, or both. It has only recently been shown that endotoxin initiates pancreatic necro-inflammation in alcohol-fed rodents[11,12] and promotes pancreatic fibrosis[12].

In healthy subjects, small amounts of endotoxin translocate from the gut lumen to the bloodstream and are naturally cleared by the reticulo-endothelial system. Under the influence of alcohol, bacteria proliferate in the small intestine[13,14], intestinal permeability is increased[15,16], while endotoxin clearance by the reticulo-endothelial system-in particular Kupffer cells in the liver - is diminished[17]. As a result, excess endotoxin is available in the blood stream and exerts its harmful effects on various organs.

This review aims to summarise the mechanisms underlying increased endotoxinemia in alcoholics, describes the role of endotoxin both as an initiating and aggravating factor of pancreatitis and attempts to define a role for the liver as a mediator in pancreatic end-organ damage.

ALCOHOL AND THE GUT FLORA

A human being harbours up to 500 different bacterial species[18], the overall bacterial cell count being 10 times more abundant than the number of eukaryotic cells in the body[19]. The combination of species-which is established during the first year of life and shaped by host genotype[20] as well as dietary factors-varies from individual to individual[21]. Moreover, there is evidence indicating that certain strains of bacteria may be unique to their host[22]. Bacterial concentrations are lowest in the upper gastrointestinal tract due to gastric acid, biliary and pancreatic secretion while the highest density of bacteria is found in the colon. In healthy humans, the gut flora prevents the growth of potential injurious bacteria[18,23], exerts metabolic activities such as the fermentation of non-digestible carbohydrates[24] or vitamin synthesis[25] and plays a role in intestinal cell growth and differentiation[26]. Several factors may influence bacterial luminal content. These include altered gut motility[27], drugs, in particular antibiotics[28] and dietary factors such as alcohol.

Alcohol has been shown to alter the jejunal microflora, since almost 50% of alcoholics with documented recent ethanol abuse displayed an increase in total number of bacteria most of which originated from the faecal flora[13]. These data were confirmed in duodenal juice samples obtained by oesogastroduodenoscopy[14] as well as H2-breath tests, as a surrogate marker of bacterial pro-

liferation in the proximal gut, in alcoholic subjects[29]. The mechanisms underlying bacterial overgrowth in alcoholism are unknown, but reduction of orocaecal transit time observed in chronic alcoholics[30,31] may offer a partial explanation. It is noteworthy, that alcohol gavage in rodents for 10 wk has the capacity to alter the composition of colonic bacteria[32].

Interestingly, certain bacteria of the gut flora have the capacity to metabolise alcohol to acetaldehyde[33,34]. In alcohol-fed rats, ethanol metabolism by colonic bacteria could be suppressed by ciprofloxacin[35] or a combination of ampicillin and neomycin[36]. In a similar animal model, administration of metronidazole increased alcohol dehydrogenase-containing bacteria and hence colonic acetaldehyde content[37]. While acetaldehyde has been measured in the rodent colon[36] and human gut bacteria have the capacity to metabolise ethanol, there is, to date, no report on acetaldehyde content of the human colon in alcoholics. Nonetheless, the above studies suggest that it would not be unreasonable to implicate acetaldehyde, as the compound that mediates most of the toxic effects of ethanol.

ALCOHOL AND GUT PERMEABILITY

In order for bacteria or bacterial products such as endotoxin to pass into the bloodstream and exert their systemic effects, they are required to cross the gut barrier. In its physiological state, the gut represents an effective barrier, made of a single continuous cell layer from the stomach to the rectum. The cells are sealed together by two sets of highly complex junctions, the more apical tight junction and the adherens junction. Physiologically, tight junctions may allow the passage of small molecules up to a molecular weight of 2000 Da but prevents the translocation of larger molecules, in particular bacterial products or bacteria[38]. In addition to this mechanical barrier, passage of bacteria or bacterial products is prevented by mucus, immunoglobulins, defensins and other antimicrobial products produced by the gut.

Intestinal permeability can be measured non-invasively using oral probes such as ethylene glycol polymers of varying molecular sizes, oligosaccharides (*e.g.*, lactulose), monosaccharides (mannitol) and radiolabeled chelates such as chromium-ethylenediaminetetraacetic acid (Cr-EDTA). All these compounds are poorly absorbed by the normal bowel mucosa and display absent or negligible metabolism. Hence, increased urinary excretion correlates with increased intestinal permeability. It is now acknowledged that the probes are absorbed *via* the paracellular route, implying that competence of the gut barrier depends on the integrity of intercellular junctions[39,40].

Several studies have addressed the question whether alcohol increases gut permeability. Early studies with rats chronically administered alcohol revealed increased permeability to macromolecules such as hemoglobin with a known molecular weight of 17 kDa[41] and horseradish peroxidase with a molecular weight of 44 kDa[42]. Permeability to smaller molecules also appears to be increased in rodents upon ethanol administration as exemplified by

increased lactulose/mannitol ratio. Increased absorption of ^{51}Cr-EDTA, a small molecule of 340 Da, was also observed in chronic alcoholics[42]. An increase in absorption of a molecule of similar size (PEG 400) was reported when alcohol was administered to volunteers with no history of chronic ethanol abuse[16]. The latter data failed to be confirmed by Parlesak *et al*[43] who did not observe a difference in the absorption of polyethylen glycol (PEG) 400 when chronic alcoholics were compared to healthy subjects. In the same study, however, permeability to larger molecules of polyethylene glycol (PEG 1500, 4000 and 10000) was significantly enhanced and the permeability to PEG 10000 in particular was 10-fold higher in alcoholics. Taken together there is experimental and clinical evidence that gut permeability is enhanced by acute and chronic ethanol administration. Permeability seems to be increased for molecules of higher molecular weight (from 1000 Da to at least 44 kDa), which is of particular relevance to the translocation of gut derived bacterial endotoxin, a large compound with a known molecular weight of 40 kDa, as a putative initiating and aggravating factor of alcohol-induced organ damage.

In order to explain increased gut permeability by alcohol, various morphological and molecular studies have been undertaken. There is evidence that alcohol exerts direct toxic effects on the gut mucosa. In an observational study by Gottfried *et al*[44], seven alcoholic subjects with a previously unremarkable oesogastroduodenoscopy were administered 1 g/kg body weight alcohol (35% w/v). Biopsy specimens taken during oesogastroduodenoscopy performed 3 h after alcohol exposure demonstrated transient focal subepithelial hemorrhage which disappeared within 3 d. These observations were corroborated by experimental data in rodents and dogs[45,46]. Studies of histological alterations in patients chronically abusing alcohol have yielded conflicting results since both histological alterations and normal mucosal structure have been described[47]. This may be related to the fact that alcohol-induced mucosal lesions are short-lived due to rapid regeneration of epithelial cells (in the study reporting normal mucosal structure, endoscopies were performed 3-14 d after alcohol withdrawal). At the molecular level, different effects of ethanol on interepithelial junctions in the gut have been described.

Ethanol at high doses has been reported to lead to increased gut permeability via direct action on tight junctions. Ma *et al*[48] measured epithelial resistance and paracellular permeability of the human adenocarcinoma cell line Caco-2 exposed to ethanol. At ethanol concentration ranging from 1% to 10% a dose-dependent drop in electrical resistance paralleled by an increase in permeability was observed. Ethanol produced a disruption of the tight junction protein ZO-1 as well as disassembly of cytoskeletal proteins such as actin and myosin. These changes proved reversible upon ethanol withdrawal. However, ethanol concentrations of 1% or above are only encountered in the duodenum/jejunum where concentrations of up to 5% have been reported[49], while ethanol concentrations in the ileum and colon tend to be much lower

(0.2%-0.25%). This would entail that most of translocation of bacteria or bacterial products occurs in the upper gastrointestinal tract.

As mentioned above, human colonic bacteria have the capacity to metabolise alcohol to acetaldehyde[33,50] *via* bacterial alcohol dehydrogenase. Accordingly, colonic acetaldehyde concentrations in the millimolar range have been observed in rats[51] and piglets[52]. Acetaldehyde concentrations of 0.1-0.6 mmol/L led to a disruption of tight junctions and adherens junction *via* tyrosine phosphorylation of their main components[53].

In summary, there is substantial evidence that alcohol increases gut permeability to large molecules of the size of endotoxin and these effects may be due to a direct toxic effect on the mucosa of the proximal gut as well as molecular modifications at the level of interendothelial junctions. Likewise, acetaldehyde, as a result of alcohol metabolism by colonic bacteria, has the capacity to disrupt epithelial junctions, suggesting that the increased serum endotoxin concentrations observed in alcoholics may also be of colonic origin.

BACTERIA AND LPS IN PANCREATITIS

In the Western society, alcohol represents 70%-80% of cases of chronic pancreatitis. As stated earlier, experimental evidence suggests that bacterial endotoxin is an initiating factor for alcoholic pancreatitis[11,12]. In addition, bacterial translocation or the passage of bacterial products such as endotoxin into the systemic circulation appears to play a primary role in systemic spread, including multiple organ system failure and prognosis of the disease[54]. While endotoxin may be a key player at both ends of the disease spectrum, *i.e.*, as an initiating and aggravating factor of pancreatitis, the mechanisms leading to its increased presence in the blood may not be the same. In this chapter, both situations will be considered separately. The question as to whether bacteria or bacterial products (LPS) translocate will be addressed first.

Sepsis, a consequence of infected pancreatic necrosis, accounts for up to 80% of deaths in severe acute pancreatitis[55]. The germs most commonly cultured from infected pancreatic necrosis are gram negative bacilli presumably as a result of increased gut permeability[55,56]. Infection of pancreatic necrosis appears to be an early event occurring within a week after initiation of the disease in more than a quarter of patients undergoing necrosectomy[55,57]. However, the translocation of entire bacteria from the gut to the systemic circulation has not been proven so far in a setting of human acute pancreatitis. Indeed, blood cultures from patients with severe acute pancreatitis are often sterile even with established infected pancreatic necrosis[58]. Ammori *et al*[54] investigated the presence of bacterial DNA in the systemic circulation of 26 patients with acute pancreatitis. No bacterial DNA was detected in any of the samples. In one patient blood cultures subsequently turned out to be positive for *E. Coli*. This study suggests that translocation of entire bacteria, as opposed to bacterial products, rarely occurs

in acute pancreatitis. However, it has to be noted that the administration of prophylactic antibiotics to 9 of 19 patients with mild attacks and all 7 patients with severe attacks of pancreatitis may have prevented significant bacterial translocation.

Endotoxin is detectable in the majority of patients with established severe acute pancreatitis, in particular in more than 90% of patients dying of the disease[59,60]. Measuring circulating anti-endotoxin antibodies Barclay *et al*[61] have observed a significant decrease in antibody titres in patients with severe acute pancreatitis compared to patients with mild disease, suggesting higher endotoxin exposure in the former. In a comprehensive study, Ammori *et al*[8] undertook to measure intestinal barrier function (by measuring intestinal permeability using a PEG probe of 3350 Da) early in the course of acute pancreatitis and to examine the correlation between intestinal permeability, endotoxinaemia and disease severity. Intestinal permeability was significantly increased in patients with severe acute pancreatitis in comparison to mild disease and disease-free controls. Changes in permeability occured early in the course of the disease, before the development of multiple organ system failure. Endotoxinaemia correlated with intestinal permeability and was present more frequently and at higher concentrations in patients with severe disease. Similar observations were made by Windsor *et al*[9] demonstrating that a significant fall in serum concentrations of immunoglubulin G antiendotoxin core antibodies as a surrogate marker for endotoxemia in patients with acute pancreatitis was predictive of pancreatitis severity and multiple organ system failure.

LPS has also been reported to be a disease modifier in experimental non-alcoholic pancreatitis induced by various treatments. In a rat model of acute pancreatitis induced by the closed duodenal loop procedure[62] disease severity was significantly worsened by endotoxin administration[62]. Pastor *et al*[63] studied the direct effect of bacterial endotoxin on the course of caerulein-induced acute pancreatitis and pancreatitis-associated lung injury in TLR4 knockout mice and TLR4 sufficient controls. Administration of LPS alone did not induce pancreatitis per se nor did it potentiate the effects of cerulein on the pancreas in either mouse strain. However, there was a significant deterioration of pancreatitis-associated lung injury when LPS was combined with cerulein in wild type mice; lung injury was significantly reduced in TLR4 knockout mice implying that the effect of LPS was mediated *via* the TLR4 pathway[63]. Surprisingly, targeted deletion of TLR4 and CD14 in mouse models of cerulein- and Arginine-induced pancreatitis without LPS administration, resulted in attenuated pancreatitis and pancreatitis-associated lung injury[64]. The latter study suggests that "endogenous" endotoxin might play a role in the pathophysiology of these models or that LPS receptors play additional roles other than LPS signal transduction in pancreatitis.

The question whether endotoxinemia is an initiating event of *alcoholic* pancreatitis, similar to alcoholic liver disease has been approached in animal models. As noted earlier, it is well known that only a minority of alcoholics will ever develop acute pancreatitis suggesting that additional factors are required to elicit overt disease. This is evidenced by experimental work in rodents where long-term administration of ethanol did not lead to pancreatitis[65]. Fortunato *et al*[11] studied the effect of intravenous LPS administration on rats fed a Lieber-de Carli liquid diet with or without alcohol. Using single LPS doses of up to 3 mg/kg body weight, the authors showed a dose-dependent increase in pancreatic lesions, while rats fed alcohol alone did not display significant pancreatic damage. In accordance with the hypothesis whereby repeated attacks of acute pancreatitis lead to chronic disease (necrosis-fibrosis sequence proposed by Ammann *et al*[66]), Vonlaufen *et al*[12] showed that repeated weekly injections of endotoxin to alcohol-fed rats led to significant pancreatic fibrosis *via* a TLR4 mediated effect on pancreatic stellate cells (PSCs), the main effectors of pancreatic fibrosis. Moreover, the presence of TLR4 and its co-receptor CD14 was detected on disease-associated and normal human pancreatic stellate cells[12,67], suggesting that PSCs are a relevant target for endotoxin in human alcoholic pancreatitis.

Taken together, endotoxin (from gut derived bacteria) appears to be an aggravating factor of pancreatitis and associated extra-pancreatic organ damage regardless of aetiology. Furthermore, there is increasing (experimental) evidence that it may play a specific role in the initiation and progression of alcoholic pancreatitis.

THE GUT-LIVER-PANCREAS AXIS

In healthy humans, trace amounts of endotoxin may transiently enter the portal circulation and are cleared by Kupffer cells in the liver. When alcohol is consumed, the detoxifying capacity of the liver seems overwhelmed, since endotoxin is detected in the systemic circulation. In 1987, Bode *et al*[3] showed for the first time that gut-derived endotoxin is increased in the systemic circulation after acute alcohol consumption by subjects with or without liver damage. The authors evaluated peripheral venous blood endotoxin concentrations in patients with alcoholic and non-alcoholic cirrhosis and in a group of alcoholics with no evidence of chronic liver disease. Increased endotoxin concentrations were found in a significantly larger proportion of patients with alcoholic liver disease (67.3%) than patients with liver disease of non-alcoholic aetiology (45.5%, $P < 0.025$). Moreover, almost half of all subjects without preexisting liver disease, presenting after a single alcoholic binge, were found to have endotoxin in the blood; importantly, in this group endotoxinemia appeared to be a transient phenomenon with no endotoxin detected after 5-8 d. Further work by the same group confirmed elevated blood endotoxin levels in a significantly higher proportion of patients with alcoholic cirrhosis compared to patients with cirrhosis of a different cause. It is noteworthy, that mean blood endotoxin concentrations were significantly higher in cirrhotics of alcoholic aetiology (19 ± 2.3 *vs* 12 ± 3.1 pg/mL, P
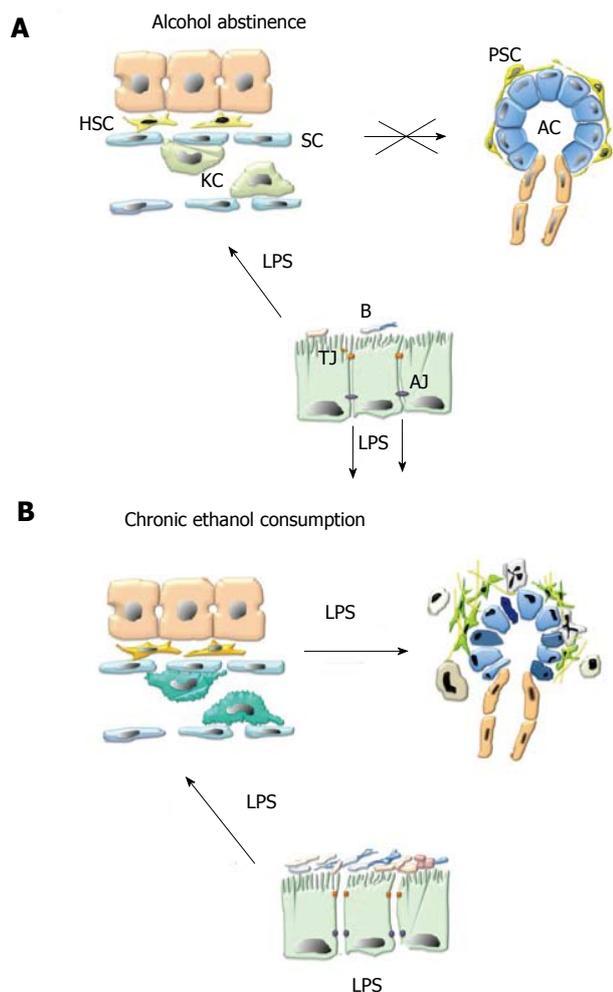
Figure 1 Alcohol and lipopolysaccharide promote pancreatic necroinflammation and fibrosis via pancreatic stellate cell activation. A: Alcohol abstinence. In healthy, non-alcoholic subjects small amounts of lipopolysaccharide (LPS) derived from the membrane of commensal gram negative bacteria (B) cross the gut epithelial barrier at the level of interendothelial junctions. LPS reaches the liver via the portal circulation where it is entirely cleared by Kupffer cells (KC) in the liver sinusoids (S), preventing it from entering the systemic circulation and reaching systemic organs such as the pancreas; B: Chronic ethanol consumption. Chronic alcohol consumption promotes bacterial proliferation in the proximal small bowel, dissociation of interendothelial junctions (by direct toxicity of alcohol and its metabolites) and leads to increased translocation of LPS into the portal circulation. In the liver, alcohol decreases the phagocytic capacity of Kupffer cells. As a result, LPS enters the systemic circulation and exerts its harmful effects on the pancreas. Alcohol and LPS promote pancreatic necroinflammation and fibrosis *via* PSC activation. TJ: Tight junctions; AJ: Adherens junctions; AC: Acinar cell; PSC: Pancreatic stellate cell.

< 0.025)[4].

Early work in patients with cirrhosis has reported toxic effects of alcohol on the reticulo-endothelial system, notably reduced phagocytic and metabolic activity of macrophages[68]. Experimentally, Kupffer cells from alcohol-fed rodents treated *in vitro* with ethanol at concentrations ranging from 10 to 100 mmol/L (corresponding to alcohol concentrations found in moderate drinkers and severe alcoholics respectively) displayed reduced endotoxin uptake and decreased production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α),

an effect that was dose-dependent[69]. Endotoxin alone activates Kupffer cells by increasing their phagocytic capacity and inducing the production of proinflammatory cytokines (such as TNF-α and interleukin-6)[70].

Whether concomitant liver disease is a co-factor for alcoholic pancreatitis remains elusive. It is well known that patients with cirrhosis are predisposed to episodes of bacterial infections, including spontaneous bacterial peritonitis with bacteria of gut origin[71,72]. Liver disease impacts on small bowel motility (and potentially bacterial overgrowth), and this effect worsens with increasing severity of liver disease[73]. Experimentally, CCl4-induced cirrhosis resulted in enterocyte oxidative stress, altered enterocyte mitochondrial function, increased lipid peroxidation and altered intestinal transport[74]. Part of the oxidative stress occurring in the enterocyte appears to be related to increased xanthine oxidase activity and increased intestinal permeability, a mechanism that can be blocked experimentally by the administration of xanthine oxidase inhibitors[75]. Accordingly, administration of allopurinol to patients with established cirrhosis efficiently reduced (systemic) oxidant stress, but did not have a significant effect on intestinal permeability[76].

Do alcoholic liver and pancreas disease occur together? A recent study by Yang *et al*[77] reviewing the epidemiology of alcohol-related pancreatic and liver disease in the United States, has reported that the prevalence of patients discharged with a diagnosis of both acute alcoholic pancreatitis and acute alcoholic hepatitis or both chronic alcoholic pancreatitis and chronic alcoholic liver disease was significantly lower than the prevalence of either disease alone. This is in conflict with necropsy data suggesting that subclinical damage to both organs often coexists[1].

PROPHYLAXIS AND SUPPORTIVE TREATMENT

Alcohol abstinence is the most obvious prophylaxis for alcoholic pancreatitis. Studies suggest that it reduces the incidence of acute attacks and retards clinical progression of the disease[78]. However, this goal is seldom reached and recurrence is common[79] (Figure 1).

Since bacteria or bacterial products appear to play a primary role in the initiation, progression and rate of complications of alcoholic pancreatitis, it appears logical to target gut bacteria either within the lumen *via* bacterial decontamination with nonabsorbable antibiotics or once translocation has occurred, *via* systemic administration of antibiotics.

Experimental evidence in rodents suggests that selective bacterial decontamination by oral, non absorbable antibiotics significantly reduced the incidence of pancreatic infection[80-82]. However, the application of prophylactic antibiotics in patients with acute pancreatitis has proven ineffective in a large randomized trial comparing the administration of meropenem *vs* placebo[83]. Another way to influence bacterial luminal content and act on gut

barrier integrity may be the application of probiotics (mostly lactobacilli or bifidobacterium strains), that is bacteria which exert protective effects on gut epithelial integrity and prevent colonization by pathogens[84]. However, in a large multicentre randomized controlled trial administration of a cocktail of probiotic bacterial strains (4 lactobacilli and 2 bifidobacteria)[85] within 72 h after onset of symptoms of pancreatitis was of no proven benefit. Moreover, excess mortality in the probiotic group was observed, with one third of deaths related to bowel ischemia. All of these patients presented with early organ failure. In a substudy it became apparent that administration of these particular probiotic bacterial strains in patients with multiple organ failure resulted in increased gut mucosal damage and permeability, as assessed by urinary intestinal fatty acid binding protein IFABP and NOx concentrations, while bacterial translocation was reduced in patients without organ failure[86].

Several animal and human studies have shown that enteral nutrition has a beneficial effect on gut mucosal integrity. In a recent meta-analysis by Petrov *et al*[87] including 5 randomised controlled trials in patients with severe acute pancreatitis, it was concluded that enteral feeding led to a significant reduction of pancreatic infections, other infectious complications and mortality, but not of organ failure. Another meta-analysis including 8 randomised controlled trials reached similar conclusions but also recorded a significant reduction in organ failure and need for surgical interventions in the total enteral nutrition (TEN) groups as compared to patients receiving total parenteral nutrition[87]. Despite overwhelming evidence in favour of early TEN in a setting of acute pancreatitis, the dogma that the diseased pancreas needs to be "put at rest" still prevails in many centers.

Taken together, early enteral nutrition significantly reduces infectious complications and mortality in patients suffering from acute pancreatitis regardless of aetiology. In contrast, the systematic administration of systemic antibiotics or of probiotics can not be recommended. To date, prophylactic studies aiming at inhibiting gut barrier dysfunction/bacterial translocation in alcoholic subjects are lacking.

CONCLUSION

There is now clear clinical and experimental evidence that bacteria and bacterial products such as endotoxin are associated with complications of pancreatitis. Furthermore, results of animal studies support the concept that bacterial endotoxin is an important factor in the initiation and progression of alcoholic pancreatitis.

Since all alcoholics may be expected to have bacterial translocation, the fact that only a minority develops overt pancreatitis indicates that genetic polymorphism plays a primordial role. Nonetheless, only two candidate genes (carboxylester lipase[88] and chymotrypsin C[89])-explaining a minoritiy of cases of alcoholic pancreatitis-have been identified so far. Additional case-control studies, comparing alcoholics with pancreatitis to alcoholics

without pancreatic disease, and targeting genes encoding tight junctional proteins or LPS-receptors are needed to clarify the issue. Moreover, particular attention should be paid to the assessment of the quality of the microbiome in these two populations.

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Molecular mechanisms of alcohol associated pancreatitis

Dahn L Clemens, Mark A Wells, Katrina J Schneider, Shailender Singh

Dahn L Clemens, Nebraska-Western Iowa Veterans Administration Medical Center, Omaha, NE 68105, United States

Dahn L Clemens, Mark A Wells, Katrina J Schneider, Shailender Singh, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198, United States

Dahn L Clemens, Shailender Singh, Fred and Pamela Buffett Cancer, University of Nebraska Medical Center, Omaha, NE 68198, United States

Author contributions: All the authors solely contributed to this paper.

Correspondence to: **Dahn L Clemens, PhD,** Department of Internal Medicine, University of Nebraska Medical Center, 4400 Emile St, Omaha, NE 68198, United States. dclemens@unmc.edu

Telephone: +1-402-9953738 Fax: +1-402-4490604

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Abstract

Alcohol abuse is commonly associated with the development of both acute and chronic pancreatitis. Despite this close association, the fact that only a small percentage of human beings who abuse alcohol develop pancreatitis indicates that alcohol abuse alone is not sufficient to initiate clinical pancreatitis. This contention is further supported by the fact that administration of ethanol to experimental animals does not cause pancreatitis. Because of these findings, it is widely believed that ethanol sensitizes the pancreas to injury and additional factors trigger the development of overt pancreatitis. How ethanol sensitizes the pancreas to pancreatitis is not entirely known. Numerous studies have demonstrated that ethanol and its metabolites have a number of deleterious effects on acinar cells. Important acinar cells properties that are affected by ethanol include: calcium signaling, secretion of zymogens, autophagy, cellular regeneration, the unfolded protein response, and mitochondrial membrane integrity. In addition to the actions of ethanol on acinar cells, it is apparent that ethanol also affects pancreatic stellate cells. Pancreatic stellate cells have a critical role in normal tissue repair and the pathologic fibrotic response. Given that ethanol and its metabolites affect so many pancreatic functions, and that all of these effects occur simultaneously, it is likely that none of these effects is "THE" effect. Instead, it is most likely that the cumulative effect of ethanol on the pancreas predisposes the organ to pancreatitis. The focus of this article is to highlight some of the important mechanisms by which ethanol alters pancreatic functions and may predispose the pancreas to disease.

Core tip: Alcohol abuse is commonly associated with the development of acute and chronic pancreatitis. Despite this close association, the fact that only a small percentage of human beings who abuse alcohol develop pancreatitis indicates that alcohol abuse alone is not sufficient to initiate clinical pancreatitis. It is widely believed that ethanol sensitizes the pancreas to injury and additional factors trigger the development of overt pancreatitis. How ethanol sensitizes the pancreas to pancreatitis in not entirely known. We will review the mechanisms by which ethanol is thought to sensitize human beings to pancreatic injury.

INTRODUCTION

The pancreas is a complex organ, containing both exo-

Alcohol abuse
sensitizes pancreases

Trigger initiates pancreatitis →

↓

Sentinel acute
pancrestitis event

Repeated episodes of pancreatic
injury (clinical or subclinical) →

↓

Alcohol-induced altered repair
and pancreatic regeneration

↓

Fibrotic scaring

↓

Chronic pancreatitis
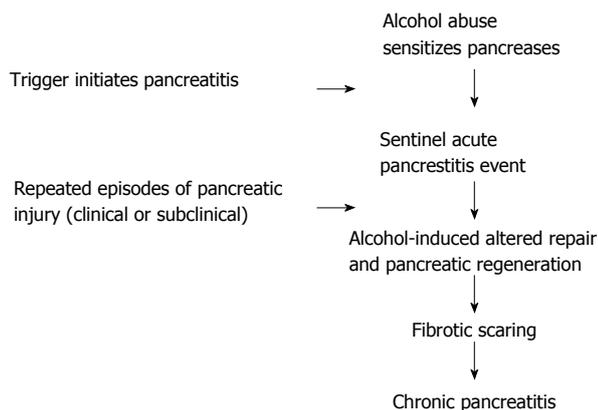
Figure 1 Proposed model for the development of alcoholic chronic pancreatitis. This proposed model incorporated alcohol abuse into the seminal acute pancreatitis event (SAPE) model proposed by Whitcomb. Alcohol metabolism results in biochemical and molecular changes in acinar cells that sensitizes the pancreas to injury. A secondary trigger initiates an initial episode of acute pancreatitis. This is the SAPE. Repeated clinical or subclinical episodes of pancreatitis coupled with ethanol-induced aberrant repair and regeneration of the damaged pancreas leads to fibrotic scarring which eventually results in chronic pancreatitis.

crine and endocrine components. The endocrine component of the pancreas comprises only about 1%-2% of the organ, and is responsible for the production of insulin and glucagon, both of which regulate glucose homeostasis. The exocrine component comprises the vast majority of the pancreas; it is composed of acinar, stellate, and ductal cells. The acinar cells produce digestive enzymes, which facilitate the digestion of carbohydrates, proteins, and lipids. The ductal cells form a network that serves as a conduit for delivery of these enzymes into the duodenum. The pancreatic stellate cells synthesize and degrade extracellular matrix proteins.

Pancreatitis, or inflammation of the pancreas, is a necroinflammatory disease of the pancreas that can manifest as either an acute or chronic disease. Acute pancreatitis is characterized by various degrees of acinar cell damage with concomitant local and systemic inflammation, mediated by inflammatory cytokines and chemokines[1]. Acute pancreatitis is usually a self-limiting condition. Unfortunately, in 10% to 20% of clinical cases, acute pancreatitis progresses to severe acute pancreatitis, a disease with high morbidity and mortality. In the United States alone there are approximately 210000 new clinical cases of acute pancreatitis a year[2]. In 2009, acute pancreatitis was the most common gastrointestinal disease requiring hospitalization. Additionally, it was estimated that acute pancreatitis accounted for more than 2.5 billion dollars in direct and indirect costs[3]. Obviously, pancreatitis is a serious public health concern.

Chronic pancreatitis is a progressive disease characterized by severe pain, persistent pancreatic inflammation, and the development of fibrotic scarring, as well as the loss of endocrine and exocrine function. It has been demonstrated in a long-term prospective study that alcoholic chronic pancreatitis normally progresses from

acute pancreatitis. Additionally, this study demonstrated that the progression of acute pancreatitis to chronic pancreatitis is associated with the frequency and severity of the acute attacks[4]. These findings are supported by the observation that individuals who suffer frequent attacks of acute pancreatitis progress to chronic pancreatitis more rapidly[5]. These findings led Whitcomb to propose that a sentinel acute pancreatitis event (SAPE) is required for the development of chronic pancreatitis[6] (Figure 1). Therefore, it appears that although acute and chronic pancreatitis have different clinical manifestations, the mechanisms by which the disease process is initiated is likely similar[7]. Unfortunately, there currently is no treatment, other than palliative care, for either of these diseases.

One of the most common factors associated with both acute and chronic pancreatitis is alcohol abuse[8]. In fact, the association between alcohol abuse and pancreatic disease has been recognized for well over 100 years[9]. It has been known for sometime that the risk of developing pancreatitis increases with increasing alcohol consumption. Recent studies have shown that a threshold of approximately 5 drinks/d (60 g of ethanol) is required for significantly increased risk of developing pancreatitis[10-12]. Although numerous studies have demonstrated direct toxic effects of ethanol and its metabolites on the pancreas, the majority of heavy drinkers (even those consuming more than 5 drinks a day) do not develop pancreatitis[8,12,13]. This fact clearly indicates that alcohol abuse itself is not sufficient to cause pancreatitis, and an additional insult or additional factors are required for the development of clinical pancreatitis. Among the factors suggested to be involved in alcoholic pancreatitis are: smoking, high fat diet, obesity, genetics, and infectious agents[12-16].

Despite the long-standing recognition of the association between alcohol and pancreatitis, the biochemical and molecular processes by which ethanol influences the initiation and progression of these diseases is not well understood. It is thought that the toxic effects of ethanol and/or the by-products of ethanol metabolism sensitize the pancreas; thereby, lowering the threshold to damage from other factors. Ethanol has been shown to affect a number of pathways and functions important in acinar cells. Alteration of these pathways may individually or cumulatively sensitize the pancreas, and lower the threshold of the pancreas to the development of overt pancreatitis. Ethanol has been shown to affect a number of pathways and functions important in acinar cells (Table 1).

Both the rapid course of acute pancreatitis and the relative inaccessibility of pancreatic tissue for examination, prior to the development of fibrotic damage in chronic pancreatitis, have hampered detailed investigations using tissue from human beings. This has contributed to our limited understanding of the mechanisms that lead to the initiation and the progression of alcoholic pancreatitis. Because of this, much of our understand-

Table 1 Mechanisms by which ethanol is thought to sensitize the pancreas to pancreatitis
Alteration of cell death pathways
Altered vesicular trafficking
Impaired autophagy
Impaired tissue repair
ER stress
Mitochondrial dysfunction

ing of pancreatitis in general, and alcoholic pancreatitis in particular, has come from the use of preclinical animal models. Preclinical models used to investigate alcoholic pancreatitis normally utilize mice or rats administered ethanol. Ethanol administration to experimental animals is commonly accomplished through the Tsukamoto-French intragastric method[17], the Lieber-DeCarli pair feeding method[18], or the Cook-Meadows model of providing ethanol in the drinking water[19,20]. Pancreatic cells are either isolated from the animals administered ethanol or pancreatitis is induced. Among the more common methods of inducing pancreatitis in these animals are: bile duct ligation, treatment with supraphysiological concentrations of the cholecystokinin (CCK) analogue caerulein, or treatment with trinitrobenzene sulfonic acid (TNBS)[21]. More recently, methods designed to be more clinically relevant have been reported. These methods include chronic ethanol administration followed by treatment with gram-negative bacterial lipopolysaccharide (LPS)[22,23], or infection with Coxsackievirus CVB3[16,24,25].

Unfortunately, no animal model of chronic pancreatitis recapitulates all of the manifestations of chronic pancreatitis in human beings. It has been demonstrated that alcohol administration to rats and mice results in acinar cell loss and enhanced fibrosis in animals subjected to caerulein-induced pancreatic injury[26,27]. Therefore, these models may be useful in elucidating the mechanisms by which ethanol alters normal pancreatic repair, and predisposes the pancreas to fibrosis.

It is the focus of this article to review and highlight some of the molecular events that may adversely affect the pancreas, and sensitize the pancreas to the initiation or progression of alcoholic pancreatitis.

ETHANOL METABOLISM

Many of the deleterious effects of ethanol are attributed to the by-products produced during its metabolism. Like the hepatocytes of the liver, the pancreatic acinar cells have the ability to metabolize ethanol by both oxidative and nonoxidative pathways. The oxidative metabolism of ethanol is catalyzed by two enzymes: the cytosolic enzyme, alcohol dehydrogenase, and the microsomal enzyme, cytochrome P450 2E1. Ethanol metabolism by both of these enzymes generates acetaldehyde and reactive oxygen species. Although the pancreas expresses both alcohol dehydrogenase and cytochrome P450 2E1, the capacity for ethanol oxidation by the pancreas is sig-

nificantly less than that of the liver[28,29]. Therefore, the actions of the oxidative metabolites of ethanol oxidation may result from both pancreatic metabolism and systemic metabolism of ethanol.

Nonoxidative metabolism of ethanol is carried out by a number of enzymes, the most important being the fatty acid ethyl ester synthases. Metabolism of ethanol by these enzymes generates fatty acid ethyl esters (FAEEs). The pancreas possesses high fatty acid ester synthase activity. Thus, the capacity for nonoxidative metabolism of ethanol in the pancreas is high[30]. In fact, a study of individuals who were intoxicated at the time of death revealed that the concentration of FAEEs in the pancreas was higher than any other organ analyzed[30]. Thus, because the oxidative metabolism of ethanol in the pancreas is relatively low, the nonoxidative metabolism of ethanol may be more important and the production of FAEEs, and their toxic effects, may be accentuated. Because the by-products of ethanol metabolism have been demonstrated to cause toxicity in other organs, a great deal of work has been performed investigating the actions of the various ethanol metabolites on the pancreas.

CELL DEATH

Cell death during an episode of acute pancreatitis can occur by one of two mechanisms: apoptosis or necrosis. The distinction between the two types of cell death not only has biological implications in the development of acute pancreatitis, but also affects the clinical presentation by influencing the severity of the illness[8]. Clinically, according to the 2012 Atlanta Classification of Acute Pancreatitis, the presence of necrosis and the number of organs affected by the subsequent inflammatory response determines the severity of acute pancreatitis (mild, moderate, severe) and dictates the short-term and long-term management of these patients[31].

While necrosis and apoptosis both lead to cell death, their respective mechanisms of achieving this end are quite different. Apoptosis, or programmed cell death, is a process by which cellular constituents are cleaved by cysteine-dependent, aspartate-directed enzymes, known as caspases. Apoptosis is mediated by caspases 3 and caspases 8. Caspase 8 is the initiator of the caspase cascade and cleaves caspase 3, which mediates many of the cellular changes that lead to apoptotic death. In pancreatitis, these caspases are activated by the release of cytochrome c from mitochondria[32]. The release of cytochrome c is caused by the depolarization of mitochondria. It appears this depolarization is a result of the opening of the mitochondrial permeability transition pore, which is caused by sustained increased calcium levels in the cytosol[33]. Ultimately, there is an organized dismantling of the cell. This leads to cell shrinkage and nuclear chromatin condensation, while preserving the integrity of the plasma membrane. Because the plasma membrane remains intact, there is very little leakage of intracellular material into the extracellular space, and therefore; there is little

activation of inflammatory cytokines.

In contrast to the organized dismantling of the cell in apoptosis, necrosis involves intracellular swelling of organelles and rupture of the plasma membrane. This results in the release of the contents of the cell into the extracellular space, which causes an inflammatory response. It has been shown in a number of preclinical animal models of pancreatitis that the severity of pancreatitis is increased with increasing necrotic cell death[8]. Additionally, perhaps the most important prognostic indicator of the severity of pancreatitis in human beings is the amount of necrosis[31].

In preclinical animal models of pancreatitis, ethanol has been shown to cause a shift in cell death from apoptosis to necrosis. This shift has been shown to occur through several mechanisms. It has been shown that the nonoxidative metabolites of ethanol, FAEEs, activate inositol trisphosphate receptors on the endoplasmic reticulum. Activation of these receptors causes release of calcium into the cytosol. As stated above, the sustained increases in cytosolic calcium results in mitochondria depolarization and loss of ATP production. Without ATP, the cells are unable to complete the apoptotic process and necrosis occurs[8].

Ethanol has also been shown to inhibit the JAK2/STAT1 pathway. Attenuated activity of this pathway leads to decreased activity of both caspase 8 and caspase 3[32]. With lower activity of these caspases, cell death by necrosis is increased while apoptotic cell death is reduced.

Ethanol also increases the pancreatic expression of cathepsin B[32]. Cathepsin B is a cysteine protease that is thought to play a major role in the intrapancreatic conversion of trypsinogen to trypsin. It has been shown that in pancreata of ethanol-fed rats, increased expression of cathepsin B result from increased levels of the transcriptional activators Ets-1 and Sp1[32]. Increases in Sp1 and Ets-1 enhance expression of cathepsin B, which leads to activation of trypsin and a shift from apoptosis to necrosis in pancreatic acinar cells[32]. These findings demonstrate that ethanol can affect the mechanism of cell death in acinar cells, and thereby influence the severity of the disease.

EFFECTS OF ETHANOL ON ZYMOGEN SECRETION

One of the primary roles of the exocrine pancreas is the synthesis and secretion of digestive enzymes. The pancreas is protected from the actions of these potentially dangerous enzymes because they are synthesized as inactive zymogens and packaged into exocytotic vacuoles, known as zymogen granules. Although ethanol has many effects on acinar cells that contribute to the development of pancreatitis, the inappropriate activation of zymogens is likely a critical component of this pathologic process.

Activation of trypsinogen is generally considered a pivotal event in the initiation of pancreatitis[34]. It has been reported by Gorlelich that treatment of isolated acinar cells with intoxicating concentrations of ethanol (25 mmol/L) sensitizes acinar cells to damage by causing the activation of zymogens[35]. The activation of these zymogens required an increase in cytosolic calcium and appeared to involve a low pH compartment (acid granular compartment).

Local cytosolic spikes of calcium in the apical region of acinar cells control the exocytotic secretion of zymogens. These spikes are generated by release of small quantities of calcium from internal stores[36]. In contrast, prolonged, global elevation of calcium results in the formation of empty looking zymogen granules, this is thought to be the site where trypsin is activated. In acinar cells treated with the FAEE palmitoleic acid ethyl ester, calcium was released from both the endoplasmic reticulum (the major calcium storage compartment of the cell) and the acid granular compartment, located near the apical surface. Additionally, it was demonstrated that the calcium release was primarily mediated by type 2 and 3 inositol 1,4,5 trisphosphate receptors[37].

Normally, zymogens are released from acinar cells by fusion of zymogen granules with the apical membrane. This fusion results in their release into the ducts, where they are transported to the duodenum and activated. The components absolutely required for membrane fusion consist of: SNAREs (soluble NSF [N-ethylmaleimide-sensitive fusion proteins] attachment proteins receptors) located on the target membrane, t-SNARES, and v-SNAREs, also known as vesicle-associated membrane proteins (VAMPs), located on the membrane of the vesicle. The t-SNARES syntaxin and synaptosome-associated proteins (SNAPs), form a SNARE complex that binds to its cognate v-SNARE; thus, juxtaposing the two membranes and facilitating the fusion of the membranes.

Interestingly, it has been demonstrated both *in vivo* and *in vitro,* that supramaximal treatment with cholecystokinin (CCK) causes basolateral exocytosis of zymogen granules in acinar cells[38]. Additionally, in both ethanol-fed rats or isolated acinar cells treated with physiologic concentrations of ethanol (20 mmol/L), stimulation with submaximal concentration of CCK or carbachol resulted in the exocytosis being redirected from the apical surface, where zymogens are normally secreted, to the basolateral surface[39]. The authors postulate that the ensuing ectopic activation of the zymogens in the interstitial space results in pancreatitis[39]. More detailed investigations demonstrated that this inappropriate exocytosis was mediated by phosphorylation of mammalian uncoordinated-18c (Munc 18c) by protein kinase C-alpha (PKC-α). Phosphorylation of Munc-18c results in its release from syntaxin-4, which is located on the basolateral surface of acinar cells. Syntaxin-4 is then able to complex with SNAP-23 and VAMP-8, located on the zymogen granules, to form the SNARE complex, which mediates the inappropriate basolateral exocytosis of zymogens[40]. Importantly, basolateral exocytosis has been

observed in tissue samples from a patient suffering from chronic alcoholic pancreatitis[41].

IMPAIRMENT OF AUTOPHAGY

Autophagy is a cellular process in which unnecessary or damaged cellular components or organelles are sequestered in vacuoles and transported to the lysosomes. Upon fusion with the lysosomes, the contents of the autophagic vacuoles, the autophagosomes, are degraded. Not only does this process perform an important role in ridding cells of unneeded components, but during times of low nutrient availability autophagy can provide the cell with needed constituents.

Impaired autophagy has been implicated in the pathogenesis of many diseases, including pancreatitis[15,42-45]. Importantly, it has been shown that ethanol can alter the process of autophagy in a number of organs, including the pancreas[43,46,47].

One of the histological hallmarks of pancreatitis is the accumulation of large vacuoles within acinar cells[48]. In a number of preclinical animal models of pancreatitis, as well as in tissue from a patient with acute pancreatitis, it has been demonstrated that these vacuoles are in fact autophagic vacuoles[44,45]. Further investigation revealed that these vacuoles possessed markers of both autophagosomes and lysosomes, and contained undegraded or partially degraded cellular material[45]. These findings indicate that at least the very late events in the autophagic process, namely the degradation of the components of the autolysosomes, are impaired during pancreatitis[45]. Thus, autophagy is activated during pancreatitis, and it appears that the impairment in the ability to complete this process is responsible for the vacuolization characteristic of this disease.

As mentioned above, trypsin activation is thought to be an early event in the initiation of pancreatitis. How this activation occurs is not well understood. It is generally thought that cathepsin B, is mis-sorted to the zymogens granules, where it co-localizes with trypsinogen. Subsequent cleavage of trypsinogen by cathepsin B results in the production of active trypsin. How trypsinogen and cathepsin B come in contact has always been a mystery. It now appears that the impairment in the completion of the autophagy may have a role in the co-mingling of these two enzymes.

Cathepsin L is an enzyme that degrades trypsinogen and trypsin, and cathepsin B is an enzyme that cleaves trypsinogen forming active trypsin. The two are important lysosomal hydrolases. During pancreatitis, increased levels of these enzymes are found in the zymogen granule fraction. Additionally, in alcoholic pancreatitis, as well as other forms of acute pancreatitis, the processing and activation of cathepsin L and cathepsin B is impaired[45,49]. Furthermore, it appears that the impairment in cathepsin L activity is more severe than the impairment in cathepsin B activity, particularly in the zymogen granule fraction[45]. Importantly, zymogen granules were

detected in the autophagosomes/autoloysosomes. The authors propose that it is in these autophagosomes/autoloysosomes that trypsinogen and cathepsin B come in contact[45]. The imbalance between cathepsin B and cathepsin L activity in these vacuoles would favor the activation of trypsin, and the initiation of pancreatitis. Thus, impairment in the completion of the autophagic process and subsequent increase in autolysosomes may contribute not only to the accumulation of vacuoles, but also to the inappropriate intracellular activation of trypsin and the initiation of pancreatitis.

Ethanol has been shown to impair other aspects of autophagy. Using a model of alcoholic pancreatitis in which rats were chronically fed ethanol and then treated with LPS to induce acute pancreatitis, Fortunato *et al*[43] demonstrated that in the pancreata of these animals fusion of autophagosomes with the lysosome was impaired. Additional studies demonstrated that Lamp-2, a lysosomal membrane protein required for the fusion of autophagosomes with lysosomes, was depleted in the pancreata of rats suffering from alcoholic pancreatitis[43,50]. Furthermore, analysis of pancreata from human beings revealed that Lamp-2 was also decreased in the pancreata of patients suffering from chronic alcoholic pancreatitis. These results indicate that the ethanol-mediated reduction in lysosomal proteins, particularly Lamp-2, and subsequent impairment in autophagy may be a contributing factor to alcoholic pancreatitis in human beings. Although not investigated, the authors speculated that disruption in the autophagic pathway may contribute to bioenergenic failure in mitochondria. Lack of mitochondrial ATP would favor necrosis, as opposed to apoptosis. Necrotic cell death would cause inflammation and lead to the initiation of pancreatitis[43].

MITOCHONDRIAL DYSFUNCTION

Pancreatic acinar cells are among the most synthetically active cells in the body[51]. This synthetic activity requires a great deal of energy. Because of this, acinar cells contain an inordinate number of mitochondria. Thus, the actions of toxins, such as ethanol, that affect mitochondria can dramatically affect acinar cells.

Normally, acetylcholine or cholecystokinin bind to G-protein linked receptors that are located on the plasma membrane of acinar cells and stimulate the production of secondary messengers. The secondary messengers bind to inositol trisphosphate or ryanodine receptors located on the endoplasmic reticulum, zymogen granules, and endo-lysosomes. This binding results in the transient release of free calcium. Mitochondria take up this calcium, which results in their activation, the synthesis of ATP, and the secretion of zymogens.

Aberrant calcium signaling has long been considered an important factor in the initiation of pancreatic injury[52]. Pathological calcium signaling in acinar cells results from prolonged global release of calcium from the endoplasmic reticulum, as well as zymogen granules and

endo-lysosomes. In fact, early acinar cell injury (vacu-olization, trypsin activation, and basolateral zymogen secretion) does not occur without prolonged, sustained release of calcium[53].

Both nonoxidative and oxidative metabolism of ethanol has been shown to contribute to mitochondrial dysfunction and acinar cell death. FAEEs, the nonoxidative metabolites of ethanol, have been shown to cause pancreatic injury by affecting calcium signaling in acinar cells[54,55]. FAEEs increase the intracellular concentration of calcium to toxic levels. This calcium increase is mediated by activation of the inositol trisphosphate receptors located on the endoplasmic reticulum, and results in global sustained increase in intracellular calcium, which causes mitochondrial membrane permeability. Mitochondrial membrane permeability can lead to cell death by either apoptosis or necrosis[56,57].

Mitochondrial membrane permeability results from opening of the mitochondrial permeability transition pore. The mitochondrial permeability transition pore is thought to have at least three major components, the voltage dependent anion channel (VDAC) located on the outer mitochondrial membrane, adenine nucleotide translocase (ANT) located in the inner mitochondrial membrane and cyclophlin-D located within the mitochondrial matrix[53].

One of the important consequences of the opening of the mitochondrial permeability transition pore and mitochondrial membrane permeability can be loss of the mitochondrial membrane potential ($\Delta\Psi M$). Loss of the $\Delta\Psi M$ results in the decreased ability of TP.

Depleted levels of ATP exacerbate the cells ability to regulate calcium by inhibiting the activity of the important ATP-dependent calcium pumps, the sacroplasmic/endoplasmic reticular calcium ATPase (SERCA) located on the ER, and the plasma membrane calcium ATPase (PMCA) located on the plasma membrane. Thus, mitochondrial membrane permeability can exacerbate the dysregulation of calcium homeostasis and lead to acinar cell necrosis.

The oxidative metabolism of ethanol also has deleterious effects on pancreatic mitochondria. Oxidative metabolism of ethanol by alcohol dehydrogenase requires oxidized nicotinamide adenine dinucleotide (NAD^+) as a cofactor, and results in the production of acetaldehyde and reduced nicotinamide adenine dinucleotide (NADH)[58,59]. Acetaldehyde is then metabolized to acetate, primarily by the mitochondrial enzyme aldehyde dehydrogenase-2. Importantly, this reaction also requires NAD^+ as a cofactor, and also results in the production of NADH[58,59]. Thus, metabolism of acetaldehyde to acetate further depletes the availability of NAD^+.

Using isolated acinar cells treated with ethanol, Shalbueva *et al*[60] demonstrated that ethanol treatment led to a decrease in the NAD^+/NADH ratio. This reduction in NAD^+ resulted in activation of the mitochondrial permeability transition pore, mitochondrial depolarization, ATP depletion, and eventually cellular necrosis[60].

Furthermore, their studies revealed that the ethanol oxidation-mediated polarization of pancreatic mitochondria was attenuated in acinar cells isolated from mice deficient in cyclophilin-D. These results indicate a role for cyclophilin-D in this ethanol metabolism-mediated mitochondrial dysfunction.

Interestingly, it has been shown in mitochondria isolated from the liver that ethanol metabolism sensitizes the mitochondrial permeability transition pore to open, in part, through increased cyclophilin-D activity and increased association of cyclophilin-D with ANT[61]. This increased activity is associated with hyperacetylation of cyclophilin-D. Acetylation of cyclophilin-D is regulated by sirtuin-3, a NAD^+-dependent deacetylase localized in the mitochondrial matrix[62]. The ethanol oxidation-mediated decrease in NAD^+ leads to decreased sirtuin-3 activity and the hyperacetylation of cyclophilin-D. Hyperacetylation of cyclophilin-D results in increased cyclophilin-D activity, increased binding to ANT, and mitochondrial permeability transition pore induction[61]. Thus, it is tempting to speculate that the ethanol oxidation-mediated induction of the mitochondrial permeability transition pore in pancreatic mitochondria is mediated by a similar NAD^+-sirtuin-3-cyclophilin-D axis.

ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

Acinar cells are responsible for the production and secretion of large quantities of digestive enzymes. Because of this, in addition to large numbers of mitochondria, acinar cells possess an extensive endoplasmic reticulum network. The endoplasmic reticulum is the major storage site of calcium in the cell, and is the cellular organelle where the proper folding and trafficking of secretory proteins is determined. Endoplasmic reticulum stress resulting from excessive accumulation of proteins, calcium imbalance, oxidative stress, or accumulation of damaged or misfolded protein leads to a response known as the unfolded protein response (UPR)[63].

One hallmark of the UPR is the activation of the IRE1/XBP1 pathway. Inositol-requiring transmembrane kinase/endonuclease 1 (IRE1) splices X-box binding protein 1 (XBP1) messenger RNA, resulting in spliced XBP1. Spliced XBP1 is a transcriptional activator that regulates a number of genes, which encode proteins that act as ER chaperones, are involved in the proper folding of proteins, or are involved in the degradation of damaged or misfolded proteins.

The UPR is activated by pancreatic injury[64]. Additionally, it has been shown that the UPR is activated in acinar cells by long-term ethanol administration to mice[65]. Ethanol mediated UPR was characterized by increased expression of IRE1 and spliced XBP1. Although the UPR was activated, ethanol administration alone did not result in histopathologic changes to the pancreas. In contrast, administration of ethanol to mice with diminished XBP1 expression (XBP1$^{+/-}$ mice) resulted in

acinar vacuolization, cell necrosis, and inflammation[65]. The presence of pathologic changes in the pancreata of XBP1^{+/-} mice led the authors to suggest that the UPR is a protective mechanism in acinar cells during endoplasmic reticulum stress. Exceeding the capacity of the UPR to compensate for endoplasmic reticulum stress results in overt pancreatitis. Thus, if the protective capacity of the UPR is exceeded, this pathway may contribute to the induction and progression of pancreatitis.

THE ROLE OF STELLATE CELLS IN ALCOHOLIC PANCREATITIS

The pancreas, like the liver, has a population of vitamin A storing cells known as stellate cells. Pancreatic stellate cells are periacinar cells located in interacinar and interlobular areas of the pancreas[66,67]. These cells are responsible for the synthesis of extracellular matrix proteins, as well as matrix metalloproteinases (enzymes that degrade extracellular matrix proteins). Thus, it appears that in the healthy organ, pancreatic stellate cells function to maintain the architecture of the pancreas by regulating the deposition and degradation of extracellular matrix components[68]. In response to pancreatic injury, pancreatic stellate cells are activated and transform into myofibroblast-like cells. Activated pancreatic stellate cells synthesize excessive amounts of extracellular matrix proteins. The accumulation of these proteins results in fibrosis. Thus, pancreatic stellate cells are intimately involved in the regulation of both normal and pathologic aspects of the pancreatitis[68,69].

Pancreatic stellate cells of both rat and human origin have the ability to metabolize ethanol through the oxidative pathway[70,71]. Rat pancreatic stellate cells possess alcohol dehydrogenase, the activity of this enzyme is induced when cells are exposed to ethanol concentrations routinely found in the blood of inebriated individuals[70]. Recently, it has also been reported that quiescent pancreatic stellate cells in human beings possess alcohol dehydrogenase activity. Additionally, this activity appeared to be upregulated in pancreatic stellate cells of individuals suffering from chronic pancreatitis and pancreatic cancer[71].

The fact that pancreatic stellate cells possess alcohol dehydrogenase activity may contribute to the development of alcoholic pancreatitis. Pancreatic stellate cells are activated when exposed to concentrations of ethanol detected in the blood of inebriated individuals (10-50 mmol/L)[70,72]. Additionally, pancreatic stellate cells isolated from both rats and human beings are activated by acetaldehyde. Ethanol and acetaldehyde not only activate pancreatic stellate cells, but also elicit responses that may have important biological consequences. Both ethanol and acetaldehyde have been shown to induce the secretion of matrix metalloproteinases in pancreatic stellate cells[73]. Furthermore, treatment of pancreatic stellate cells with ethanol induces the synthesis of interleukin-8 and connective tissue growth factor (CTGF)[72,74]. It has

been suggested that these factors act in an autocrine manner to perpetuate the activation of pancreatic stellate cells[13]. This finding may help to explain both the apparent inability of the pancreas to fully recover from injury in the continued presence of ethanol, and the extremely common association between alcohol abuse and chronic pancreatitis.

Although it is well established that pancreatic stellate cells are primarily responsible for the deposition and degradation of components of the extracellular matrix, it appears that acinar cells exposed to ethanol may also contribute to the increase in extracellular matrix deposition. It has been shown that FAEEs can increase the levels of extracellular matrix proteins by inhibiting the acinar cell activity of plasmin and urokinase-type plasminogen activator (uPA) proteins involved in the degradation of the extracellular matrix components[75].

THE ROLE OF THE INFLAMMATORY RESPONSE

Inflammation mediated by cytokines, chemokines, and adhesion molecules is involved in the development of pancreatitis[1,76,77]. Interestingly, it appears that ethanol and its metabolites have a differential effect on the expression of molecules that regulate the inflammatory response. It has been shown that treatment of isolated acini with ethanol or acetaldehyde decreased the activity of two important transcriptional activators involved in the inflammatory response, specifically nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). Conversely, treatment of acini with FAEEs increased the activation of these regulators of the inflammatory response[78].

The activity of NF-κB is also reduced in the pancreata of animals chronically fed ethanol[79]. However, it was demonstrated that induction of pancreatitis in rats chronically administered ethanol resulted in increased NF-κB activity, as well as increases in the mRNA levels of a number of proinflammatory cytokines, including: tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), macrophage inflammatory protein-1 (MIP-1), and monocyte chemotactic protein-1 (MCP-1)[79]. These results led the authors to suggest that the *in vitro* and *in vivo* down-regulation of these factors by ethanol reflected a protective mechanism to prevent the development of alcohol-induced pancreas[78,79].

The role of the inflammatory response in chronic alcoholic pancreatitis has also been investigated[80]. Focusing on the resident mononuclear cells of the pancreas, Deng *et al*[80] demonstrated that chronic ethanol administration reduced the number of these cells present in the pancreas. In agreement with others, they suggested that this reduction likely reflected a general immunologic suppression in the pancreas of ethanol-fed rats, and may explain why animals chronically provided ethanol do not develop chronic pancreatitis in the absence of acute pancreatic damage[80].

Despite this immunologic suppression, when pan-

creatitis was induced by caerulein, the inflammatory response in these animals was enhanced[80]. Furthermore, following repeated caerulein-induced episodes of pancreatitis, it was shown that the expression of both pro-inflammatory cytokines such as TNF-α, MIP-1α, and RANTES (regulated on activation normal T cell expressed and secreted), as well as the anti-inflammatory cytokines tissue growth factor-β (TGF-β) and interleukin-10 (IL-10) was enhanced. The increase in cytokine expression was only observed in rats fed ethanol and subjected to repeated episodes of acute pancreatitis, and was also associated with increased activation of pancreatic stellate cells and fibrosis. These findings led the authors to suggest that ethanol acts not only to sensitize the pancreas to acute pancreatitis, but also aids in the progression of chronic pancreatitis if repeated episodes of acute pancreatitis occur[80].

EFFECTS OF ETHANOL ON PANCREATIC REPAIR

It is generally accepted that fibrosis is an aberrant repair response. It appears that in the presence of ethanol, repair of the damaged pancreas is altered or never fully completed[26,27]. This may help to explain the extremely common association between alcohol abuse and chronic pancreatitis. Because ethanol and acetaldehyde can activate stellate cells, and FAEEs inhibit the degradation of extracellular matrix proteins, it is obvious that ethanol can also influence recovery of the pancreas after damage has occurred[70,72,75].

It has been demonstrated that chronic ethanol administration also delays regeneration of the damaged pancreas[81]. This delay was associated with an ethanol-mediated decrease in the expression of important developmental factors, such as PDX-1 and PTF-1a, as well as impaired activation of the Notch signaling pathway[24]. Normal pancreatic repair requires the dedifferentiation of mature acinar cells followed by their redifferentiation[82]. Thus, ethanol-mediated alterations in the expression of these important developmental factors affect the dedifferentiation/redifferentiation of acinar cells. These alterations may dramatically influence pancreatic repair.

As mentioned above, there is a close association between alcohol abuse and chronic pancreatitis. In fact, in developed countries, alcohol abuse is associated with over 70% of the reported cases[83]. Importantly, individuals suffering from chronic pancreatitis have a 20-fold greater likelihood of developing pancreatic cancer[84], a disease with a dismal prognosis. It is thought that changes that occur in the pancreas during chronic injury are associated with, or predispose the organ to, the initiation of pancreatic neoplasia. Because one of the seminal characteristics of chronic pancreatitis is aberrant tissue repair, resulting in fibrotic scarring, and ethanol consumption alters pancreatic repair, ethanol may have an indirect role in the initiation of pancreatic cancer. Thus, the effects of ethanol on repair of the damaged pancreas may be a contributing factor in pancreatic cancer, as well as alcoholic pancreatitis.

CONCLUSION

Despite the dramatic expansion of our understanding of pancreatitis in general, and how ethanol and its metabolites affect pancreatic cells, we still have not defined the mechanism of alcoholic pancreatitis. Instead, it is evident that ethanol has a plethora of toxic affects on pancreatic cells. Because all of these effects occur simultaneously, it is likely that the cumulative effects of ethanol sensitize the pancreas to damage, and that "alcoholic pancreatitis" is a multifactorial disease. Paradoxically, despite the demonstration that ethanol has numerous toxic effects on the pancreas, data from demographic studies and preclinical animal models has firmly established that ethanol itself does not cause pancreatitis. Because ethanol does not cause pancreatitis, but only sensitizes the pancreas to disease, it appears that the pancreas has developed protective mechanisms that can partially compensate for ethanol-induced cellular damage. Some of these protective mechanisms have been identified. It is likely that additional compensatory mechanisms exist. Further defining the mechanisms of ethanol-induced pancreatic injury may help define these protective mechanisms. It is hoped that this strategy will lead to the development of therapeutic targets that will prevent or reduce the severity of alcoholic pancreatitis.

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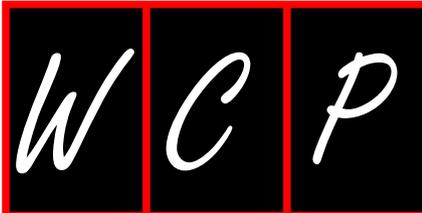
Early phase of acute pancreatitis: Assessment and management

Veit Phillip, Jörg M Steiner, Hana Algül

Veit Phillip, Jörg M Steiner, Hana Algül, II. Medizinische Klinik und Poliklinik, Klinikum rechts der Isar der Technischen Universität München, 81675 München, Germany

Jörg M Steiner, Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A and M University, College Station, TX 77843-4474, United States

Author contributions: Phillip V and Algül H collected and analyzed the literature; Phillip V prepared the original draft; Steiner JM and Algül H supplemented the material and revised the manuscript; all authors approved the final version of the paper to be published.

Correspondence to: Hana Algül, MD, MPH, II. Medizinische Klinik und Poliklinik, Klinikum rechts der Isar der Technische Universität München, Ismaninger Straße 22, 81675 München, Germany. hana.alguel@lrz.tum.de

Telephone: +49-89-41405215 Fax: +49-89-41406794

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Abstract

Acute pancreatitis (AP) is a potentially life-threatening disease with a wide spectrum of severity. The overall mortality of AP is approximately 5%. According to the revised Atlanta classification system, AP can be classified as mild, moderate, or severe. Severe AP often takes a clinical course with two phases, an early and a late phase, which should both be considered separately. In this review article, we first discuss general aspects of AP, including incidence, pathophysiology, etiology, and grading of severity, then focus on the assessment of patients with suspected AP, including diagnosis and risk stratification, followed by the management of AP during the early phase, with special emphasis on fluid therapy, pain management, nutrition, and antibiotic prophylaxis.

Key words: Acute pancreatitis; Incidence; Pathophysiology; Etiology; Severity; Risk stratification; Fluid therapy; Pain management; Nutrition; Antibiotic prophylaxis

Core tip: Acute pancreatitis is a frequent and potentially life-threatening disease. Therapy is currently mostly symptomatic with fluid resuscitation, pain management, and early oral feeding. Vigorous fluid resuscitation remains a cornerstone of early management of acute pancreatitis. Cross-sectional imaging during the early phase of evaluation has not been associated with improvement in outcome. There is no role for prophylactic antibiotics in the management of the early phase of acute pancreatitis (AP). Enteral nutrition in AP can reduce mortality, systemic infections, and multiorgan dysfunction compared to parenteral nutrition. Immediate endoscopic retrograde cholangiography is indicated only in patients with biliary pancreatitis with common bile duct obstruction and cholangitis.

INTRODUCTION

Acute pancreatitis (AP) is a potentially life-threatening disease with a wide spectrum of severity. The reported incidence of acute pancreatitis differs depending on geographic location and ranges from 14.7/100000 person years in the Netherlands to 45.1/100000 person years in Japan[1,2]. However, most studies show an incidence between 30 and 45/100000 person years[2-7]. Many studies report an increase in incidence over the last few decades[2,3,8], however, it is a matter of debate whether this

represents a real increase in incidence due to increasing biliary AP in an increasingly obese population or whether this rise in incidence is due to improved diagnostic capabilities, a higher level of suspicion of this disease, or an overestimation of retrospective studies using administrative diagnostic codes[9-11]. In 2009, AP was the most common principal gastrointestinal diagnosis at discharge in the Unite States with estimated inpatient costs of $2.6 billion per year. Furthermore, it was the 14th most common cause of death with a crude rate of 1.0 per 100000 inhabitants[12]. The overall mortality of AP is about 5% and can reach up to 20%-30% in patients with severe AP and infected necrosis[13,14]. While there seems to be an increase in incidence, several studies have reported a decrease in mortality. Again, this could be a real decrease due to an earlier diagnosis and better therapeutic options or it may also be due to an improved sensitivity of diagnostic modalities, leading to an increase in the diagnosis of mild forms of pancreatitis[15].

In this review article, we first discuss general aspects of AP, including pathophysiology, etiology, and grading of severity, then focus on the assessment of patients with suspected AP, including diagnosis and risk stratification, followed by the management of AP during the early phase with special emphasis on fluid therapy, pain management, nutrition, and antibiotic prophylaxis.

PATHOPHYSIOLOGY

The pathophysiology of AP with multi organ failure (MOF) is poorly understood. Researchers have long hypothesized that AP results from premature activation of digestive enzymes within the pancreas, a process referred to as autodigestion. Indeed, inherited mutations in genes encoding for digestive enzymes have been found in patients with a hereditary form of pancreatitis[16]. However, affected patients develop chronic, rather than acute pancreatitis. Therefore, in recent years, a novel concept has evolved, suggesting that systemic complications during AP result from uncontrolled activation of the inflammatory cascade. As indicated above, severe AP is associated with a significant mortality. Thus, early identification of severe forms of AP is crucial for outcome. In an attempt to identify surrogate parameters as predictors for severe AP, several association studies linking cytokines and chemokines with AP severity have been conducted[17]. Among these, serum levels of interleukin (IL)-6 and the IL-6-dependent acute phase protein, C-reactive protein (CRP) were identified as the most reliable predictors for severe AP[18,19]. Recent results from basic research have established that IL-6 or CRP are not only relevant markers to predict the severity of AP, but that the cytokine IL-6 also has a substantial pathophysiological impact on the course of the disease[19]. While excessive stimulation of the inflammatory cascade [hyper-inflammatory state, systemic inflammatory response syndrome (SIRS)] accounts for early systemic complications, paralysis of the inflammatory response, also termed compensatory anti-

inflammatory response syndrome (CARS), contributes to local complications and sepsis associated with the late phase of the disease. Although these definitions are largely non-specific, they are undeniably useful in the clinical and research setting. Among the agents contributing to this anti-inflammatory response, IL-10 may be of importance. In fact, the protective role of IL-10 in experimental studies in animal models has been well documented[20]. Thus, the hypo-inflammatory status of CARS might facilitate superinfections that lead to extensive necrosis and/or septic complications. This interplay of these two contrasting phenomena requires an individualized therapeutic approach[20-22].

ETIOLOGY

The identification of the etiology of AP is crucial for the management during the early phase of the disease and also for the prevention of recurrence of AP. Although there is no specific therapy for AP, the causing factor, *e.g.*, choledocholithiasis in biliary AP, must be investigated and eliminated if identified. The most common causes of AP are gallstones and prolonged heavy use of alcohol, which together account for about 60%-80% of all cases. The incidence of biliary etiology differs considerably between different geographic regions. For example, there is a clear predominance for biliary AP over alcoholic AP in Greece (71.4% *vs* 6.0%) whereas the opposite is the case for Finland (6.3% *vs* 79.3%)[23,24]. The regional differences in frequency of biliary and alcoholic etiology are shown in Figure 1[6,7,23-31].

Other causes of AP include ERCP (0.4% to 11%[32,33]), idiosyncratic reactions to drugs (0.1% to 2%)[34], hypertriglyceridemia (1.1%-3.8%)[6,23,35], anatomic alterations[36], genetic predispositions[37], and other rare causes[38,39]. Despite a thorough clinical workup, 10%-25% of all cases remain idiopathic[6,11,23,33].

NATURAL COURSE OF ACUTE PANCREATITIS

The severity of AP can be subclinical, mild without organ dysfunction, or can be severe. Patients with mild disease often improve spontaneously and heal within a few days. However, patients with severe disease may develop life-threatening local and/or systemic complications. According to the revised Atlanta classification system, AP can be classified as mild, moderate, or severe[40]. However, it is important to remember that AP is a rapidly evolving, dynamic condition in which the severity may change rapidly during the course of the disease[40]. Severe AP often takes a clinical course with two phases, an early and a late one, which should both be considered separately[40].

The early phase, which usually lasts for about one week, is characterized by a complex inflammatory reaction. The course of AP starts with a systemic proinflammatory phase systemic inflammatory response syndrome
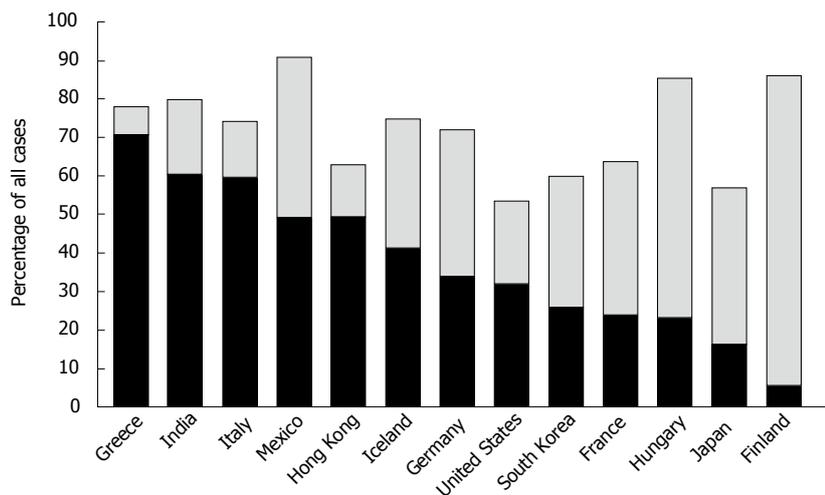
Figure 1 **Regional differences in frequency of biliary (black) and alcoholic (gray) etiology of acute pancreatitis.**
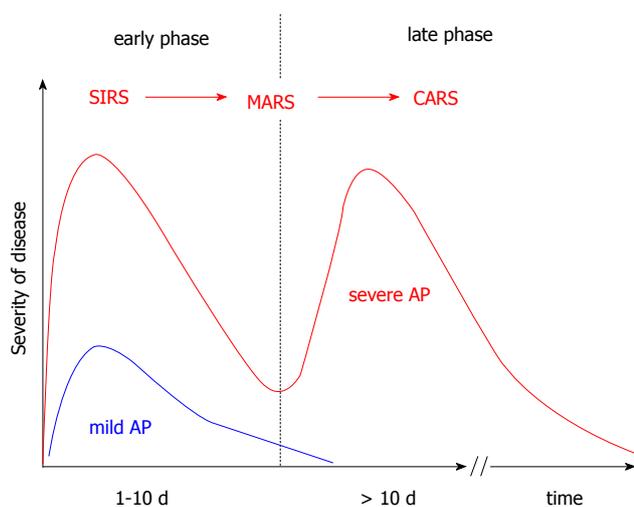

Figure 2 **Two-phase course of severe acute pancreatitis.** CARS: Compensatory anti-inflammatory response syndrome; MARS: Mixed anti-inflammatory response syndrome; SIRS: Systemic inflammatory response syndrome.

(SIRS), followed by a mixed inflammatory response syndrome mixed antagonist response syndrome (MARS), and finally leads to a phase with a suppressed inflammatory response compensatory anti-inflammatory response syndrome (CARS)[41-43]. In the phase of CARS, the immune system is downregulated and the chance of an infection of pancreatic and peripancreatic necrotic tissue rises. This is likely the reason why infections usually do not occur earlier than at the end of the first week[44]. During the stage of CARS pathogens can migrate unopposed from the intestinal lumen into necrotic tissue in and around the damaged pancreas. At that point, the clinical course of AP moves towards the second phase, including SIRS, sepsis, local and systemic complications, persistent organ failure, and possibly death. The model of the two-phase course is shown in Figure 2.

Efforts must be made to predict the severity of the disease as early as possible in order to know whether a patient diagnosed with AP can be treated as an outpatient, has to be admitted to a regular ward, to an intermediate care facility, or even to the intensive care unit. While it is generally recognized how important the prediction of severity of the disease is for the management of the individual patient, it is also recognized that such prediction is very difficult. Underestimation of the severity could be harmful for the patient, while overestimation could lead to unnecessary costs and a waste of resources. Therefore, the assessment and prediction of the severity is crucial for the management of the disease. A lot of research has been done over the last few decades trying to identify new tools to accurately predict the severity of pancreatitis, yet no gold standard for such prediction of the course of AP has been identified. An ideal predictor should be fast and easy to obtain, widely available, economical, and associated with a high sensitivity and specificity. Even though there are several clinical scores with a high sensitivity, specificity, positive, and/or negative predictive value, many of them are complicated to asses or can predict severity only after 48 h of admission to the hospital, which effectively means more than 72 h after the onset of disease[45]. This might be too late, as early aggressive fluid resuscitation is a cornerstone of AP therapy.

ASSESSMENT

Diagnosis
The diagnosis of AP can be made if ≥ 2 of the following three criteria are fulfilled: (1) abdominal pain characteristic of acute pancreatitis; (2) elevation of serum lipase or amylase activity > 3-fold of the upper limit of the reference interval; and (3) characteristic signs of pancreatitis on computed tomography (CT) imaging.

The first step in the diagnosis of AP should be a thorough clinical history. The pain caused by AP is typically dull, located in the epigastrium, may radiate into the back, and is usually severe, leading to hospital admission and often necessitating opioid therapy[45,46]. Furthermore, AP often causes nausea and vomiting. Known cholecystolithiasis and/or colics, alcohol excess within 48 h before the onset of pain, new medications, and the character of the pain should be evaluated. The second step of pancreatitis diagnosis is based on clinical

Table 1 Prognostic criteria of Ranson	
On admission	**After 48 h**
Age > 55 yr	Hematocrit fall > 10%
White blood cell count > 16000/mL	BUN increase > 1.8 mmol/L
Blood glucose concentration > 11.1 mmol/L	Serum calcium < 2 mmol/L
LDH > 350 IU/L	PaO₂ < 60 mmHg
ASAT > 250 IU/L	Base deficit > 4 mmol/L
	Fluid sequestration > 6 L

ASAT: Aspartate aminotransferase; BUN: Blood urea nitrogen; LDH: Lactate dehydrogenase; PaO$_2$: Partial pressure of arterial oxygen.

Table 2 Bedside index of severity in acute pancreatitis score and observed mortality by bedside index of severity in acute pancreatitis score score	
BUN > 8.9 mmol/L	
Impaired mental status (Glasgow coma scale < 15)	
SIRS, defined by the presence of two or more	
Temperature	< 36 ℃ or > 38 ℃ (< 96.8 °F or > 100.4 °F)
Heart rate	> 90 per minute
Respiratory rate	> 20 per minute or PaCO$_2$ < 32 mmHg
White blood cell count	< 4000/mL or > 12000/mL or > 10% immature neutrophils
Age > 60 yr	
Pleural effusion	
BISAP score	**Mortality (%)**
0	0.1-0.2
1	0.5-0.7
2	1.9-2.1
3	5.3-8.3
4	12.7-19.3
5	22.5-26.7

BUN: Blood urea nitrogen; PaCO$_2$: Partial pressure of arterial carbon dioxide; SIRS: Systemic inflammatory response syndrome.

chemistry. The measurement of serum lipase activity is generally thought to be more sensitive and specific than that of serum amylase activity and there is no additional value in simultaneous measurement of serum lipase and amylase activities[14,47]. Also, the degree of the elevation of serum pancreatic enzyme activities does not correlate with the severity of the disease, although, some studies would suggest such a correlation between serum enzyme activity and severity[6,45]. Only in patients with characteristic epigastric pain, but serum enzyme activities below 3-fold of the upper limit of the reference interval, a CT scan should be considered to rule out other differential diagnoses or to confirm AP. Apart from that, a CT in the early phase of AP is not recommended by current practice guidelines[14,48,49].

Risk stratification

Risk factors: Obesity favors the development of local and systemic complications in patients with AP[50]. Since assessment for obesity is simple and free it should be assessed in every patient. The same applies for age, as patients 55 years or older are at increased risk for severe disease[14].

Scoring systems: Several single parameters and more or less complex scoring systems for the prediction of the severity of AP have been developed and clinically evaluated and all of them have been shown to be associated with advantages and disadvantages. The HAPScore (harmless acute pancreatitis score) was developed to identify patients with mild AP who can be treated as outpatients. Patients without rebound tenderness and/or guarding, a normal hematocrit, and a normal serum creatinine concentration have a high probability (positive predictive value: 98%-98.7%) to have a harmless course of the disease[51,52].

One of the oldest and probably best known and heavily used scores to predict a severe course of pancreatitis was developed in the early 70ties by John Ranson and colleagues[53]. The Ranson score is based on the presence or absence of simple parameters and is assessed differently at the time of admission (5 parameters; possible scores: 0-5) and 48 h later (6 parameters; possible scores: 0-6; Table 1).

Although a score ≥ 3 has a high sensitivity and spec-

ificity regarding a severe course of pancreatitis (83.9% and 78.0%, respectively) and a negative predictive value of 94.5%, the severity can be predicted no earlier than 48 h after admission[25,54]. A modification of the Ranson score by Clemens Imrie and colleagues (Imrie score or Glasgow score) was first reported in 1978 and is still widely used and has a similar accuracy as the Ranson score[25,55].

Currently, the score with the highest sensitivity regarding prediction of a severe course is the Acute Physiology And Chronic Health Evaluation (APACHE) Ⅱ score[14,56]. Originally developed to predict mortality in intensive care patients, a value ≥ 8 of the APACHE Ⅱ score predicts a severe course of AP with a sensitivity of 65%-83%, specificity of 77%-91%, positive predictive value (PPV) of 23%-69%, and negative predictive value (NPV) of 86%-99%[54,57]. However, the determination of an APACHE Ⅱ score in a clinical patient is complex and time-consuming as it utilizes more than 15 parameters, which limits the clinical value of this score.

A score that was developed and validated more recently in almost 18000 patients, is the BISAP (Bedside Index of Severity in Acute Pancreatitis) score[58]. The main advantage of the BISAP score is its simplicity. One point each is given for blood urea nitrogen (BUN) > 8.9 mmol/L, impaired mental status (Glasgow Coma Scale < 15), presence of SIRS, age > 60 years, and pleural effusion (Table 2). A score ≥ 3 is predictive for a severe course (observed mortality of > 5%; Table 2) with a sensitivity of 83% and a PPV of 76.9%[58-60]. One disadvantage of the BISAP score is, that this score cannot easily distinguish patients with transient and persistent organ failure and therefore may overestimate severity and preclude differentiation between moderate and severe AP.

In summary, there is currently no ideal predictor of severity of AP. All prognostic factors and scores show a

Table 3	Balthazar score
Grade A	Normal pancreas
Grade B	Focal or diffuse enlargement of the pancreas
Grade C	Pancreatic changes associated with peripancreatic inflammation
Grade D	Single fluid collection
Grade E	Two or more fluid collections and/or presence of gas within the pancreas or within peripancreatic inflammation

Table 4	Computed tomography severity index	
Extent of necrosis		**Points**
Absence of necrosis		0
< 30% necrosis		2
30%-50% necrosis		4
> 50% necrosis		6
Balthazar score		
A		0
B		1
C		2
D		3
E		4

Maximum score 10 points.

good NPV, but suffer from a low PPV. Thus, the main value of severity assessment is to exclude a large number of patients with a low risk of mortality[57].

In addition to the laboratory/clinical scoring systems described above there are scoring systems based on imaging results to assess and predict the severity of AP. A CT scan for diagnostic purposes and severity assessment has been-and probably still is-standard practice in many centers[61]. The Balthazar score, developed in 1985, categorizes patients with AP into 5 groups (A-E) according to pancreatic and peripancreatic changes diagnosed by CT (Table 3)[62]. In 1990, Balthazar *et al*[63] modified this score, including assessment of the extent of pancreatic necrosis and named this score Computed Tomography Severity Index (CTSI) (Table 4). The CTSI is probably the most frequently used imaging score to assess severity in patients with AP and a score ≥ 4 has a negative predictive value of 94%-97% and a positive predictive value 53%-69% regarding the clinical severity of disease[61,64].

In addition to the Balthazar score and the CTSI, several other scores, *e.g.*, pancreatic size index (PSI), mesenteric edema and peritoneal fluid (MOP) score, extrapancreatic (EP) score, extrapancreatic inflammation on CT (EPIC) score, modified CTSI (MCTSI), and MR severity index (MRSI) have been developed and evaluated[61,65]. However, none of these imaging scores were shown to be superior to clinical scoring systems. Thus, a CT on admission to predict severity of AP cannot be recommended at the current time[61].

In addition to laboratory/clinical and imaging scoring systems, single parameters have been evaluated to assess and predict severity.

A lot of research has been done evaluating hematocrit as an indicator for hemoconcentration. The first prospective cohort study showed a high NPV for a hematocrit ≥ 44 % (93% on admission and 97% 24 h later) but a poor PPV (26% and 27%, respectively) regarding organ failure in AP[66]. Similar results were obtained by several other studies focusing on the usefulness of hematocrit to predict a severe course of AP, organ failure, pancreatic necrosis, or death[67,68]. Due to its high negative predictive value, its low cost, and the ease of measurement, the hematocrit has value in predicting a non-severe course of AP.

The disruption of water balance can lead to hypoperfusion and a disturbance of pancreatic microcirculation[69], which in turn correlates with the severity of AP[70,71]. Understanding the water balance and the result-

ing changes in laboratory tests can help to predict severity and outcome of AP. In addition to hematocrit, other parameters, that mirror intravascular volume depletion, can also be helpful.

Serum creatinine has been identified as a predictor for pancreatic necrosis. Also, more recently, an estimated glomerular filtration rate (GFR) < 90 mL/min per 1.73 m^2 on admission has been shown to predict pancreatic necrosis with a sensitivity, specificity, PPV, and NPV of 78.1%, 71%, 64%, and 83%, respectively[72,73]. While only one study has described GFR as a predictor of severity, BUN has been evaluated for many years and has been shown to be a good predictor for severity in AP in several large studies. A rise in BUN > 1.8 mmol/L after 48 h had already been included in the Ranson score 40 some years ago, is one of the 4 parameters used in the BISAP score, and has also been shown to have a high predictive value as a single parameter[74,75].

Besides parameters focusing on water balance and microcirculation, laboratory parameters suggesting the presence of an inflammatory process have been used as a predictor of severity. The most intensively studied parameter is CRP. In one study, a serum CRP concentration of 150 mg/L or greater predicted severe AP at 36 h after admission with a sensitivity, specificity, PPV, and NPV of 86%, 87%, 75%, and 93%, respectively[76]. However, the prediction of severity was only possible more than 24 h after admission, which, on average, is about 50 h after the onset of pain[45]. Also, several other studies showed a high predictive value of CRP during the course of AP in regards to severity, but a very low predictive value on admission[77,78].

Procalcitonin appears to be a valuable tool to discriminate between sterile and infected necrosis within the first days of AP[79,80]. However, data on the ability to predict the course of AP are not consistent. On one hand, a multicenter study from the United Kingdom found a significant difference of procalcitonin concentrations measured within 48 h of the onset of symptoms in patients with mild and severe AP and showed an accuracy of 94% in predicting death[81]. In a study from Slovakia, the PPV for predicting a fatal outcome reached 75% when a cut-off value of 5 ng/mL was used[82]. A

third study evaluating procalcitonin showed an accuracy of 76% and a PPV of 75% for predicting a severe course of pancreatitis[83]. On the other hand, two studies reported that procalcitonin is not useful in predicting the severity of AP upon admission[79,84]. However, the time point for determination of procalcitonin concentrations, the assays used, and the cut-off values applied were different for all studies. Finally, measurement of procalcitonin is not widely available and is expensive.

A blood glucose concentration < 6.9 mmol/L on admission has a high negative predictive value (92%) for pancreatic necrosis and also can serve as a predictor for severity[85,86]. Blood glucose is easy, fast, and inexpensive to determine and widely available and therefore should be included in the risk stratification.

In summary, there is no single marker that can adequately predict the severity of AP, but there are several scoring systems that can be used to assess and predict the severity of AP. However, these scoring systems must be applied at the correct time, the correct place, and in the correct patient. Also, it is important to observe patients carefully and reassess severity frequently as the disease course can change rapidly at any given time.

MANAGEMENT

Patients diagnosed with mild AP (according to the HAPScore) and no other risk factors can be treated as outpatients. In contrast, patients with any of the above-mentioned risk factors should be considered for admission to the hospital for close monitoring and timely reassessment of disease severity. In contrast, patients with a Ranson score ≥ 3, a BISAP score ≥ 3, an APACHE-II score ≥ 8, or patients with apparent organ failure should be transferred to an advanced medical care ward or facility.

Therapy

Fluid therapy: Despite a lot of research, there is no pharmacological treatment of AP[87]. Thus, fluid resuscitation, analgesia, supportive care, and management of the local and systemic complications are the key elements of the management of patients with acute pancreatitis. One of the most important components of therapy of AP is early intravenous fluid resuscitation[88]. In fact, the decrease in mortality observed over the last decade might be due to the prevention of pancreatic necrosis by maintenance of microcirculation due to more aggressive fluid resuscitation[89]. Two studies have shown a decrease in mortality by early and aggressive fluid resuscitation[90,91]. However, data on the amount of fluid needed to prevent necrosis or to improve outcome are contradictory and the volume must be adjusted to the patient's age, weight, and pre-existing renal and/or cardiac conditions[92]. The importance of starting fluid resuscitation as early as possible and in fact already in the emergency room was shown by two retrospective studies[90,91]. However, the optimal type of fluid is still a

matter of debate. Studies comparing isotonic saline and lactated Ringer's solution and crystalloid *vs* colloid solutions, respectively, showed no differences between both groups regarding clinical outcome as determined by the frequency of pancreatic necrosis, length of hospital stay, or mortality[93,94]. Also, the optimal therapeutic goal of fluid resuscitation is not yet clear. A goal-directed fluid resuscitation algorithm based on changes in BUN measurements, as a mirror of renal function, showed no improvement in outcome in patients with AP[93]. Nonetheless, blood pressure, respiratory function, urine output, and-where appropriate-intraabdominal pressure should be closely monitored. One study showed a less severe course of post-ERCP pancreatitis when patients were treated according to a fluid resuscitation protocol based on vital signs and hematocrit[95]. While questions on the type of fluid, the optimal rate of administration, and the therapeutic goal to reach remain unanswered[96], the time-point appears to be very important - the earlier, the better[90,91].

Causative therapy: Elimination of any potential risk factor is another important approach to AP therapy. In case of suspected alcohol- or drug-induced AP, the intake of the causing agent must be stopped immediately. In case of biliary AP, the indication to perform an endoscopic retrograde cholangiography (ERC) and removal of stones within the bile duct depends on the degree of obstruction of the common bile duct and the presence of cholangitis. Biliary pancreatitis and cholangitis are clear indications for ERC and ERC should be performed as early as possible[49,97,98]. Immediate ERC is indicated in patients with biliary pancreatitis with common bile duct obstruction and cholangitis, arguable in patients with predicted severe pancreatitis but without cholangitis, and not indicated in predicted mild pancreatitis without cholangitis[49].

After biliary pancreatitis, cholecystectomy is recommended within the same hospital stay for mild pancreatitis or after an interval of 6 wk following an episode of severe pancreatitis[49].

Pain management: Given that most patients with AP suffer from severe pain, adequate analgesia is very important. In mild cases, non-opioid drugs might be satisfying, but in many cases, especially severe AP, parenterally administered narcotic agents are warranted and most patients will require the use of opioids to control the pain[99,100]. In contrast to historical reports, there is no evidence or a recommendation for restrictions on the type of pain medications being used[14].

Nutrition: For many years, resting the pancreas by giving the patient nothing per os was an important part of therapy. Nowadays, there is wide agreement that total oral abstinence from food combined with total parenteral nutrition is not beneficial to patients with severe AP, but may in fact be detrimental. A recent meta-analysis

showed a statistically significant association of early enteral nutrition and reductions in systemic infections, pancreatic infections, length of hospital stay, and mortality[101]. Also, in patients with severe AP, enteral nutrition was significantly superior to total parenteral nutrition regarding mortality, infectious complications, and organ failure[102]. Gut barrier function is compromised in patients with acute pancreatitis, likely leading to bacterial translocation and potentially causing infected necrosis or even sepsis[103,104]. Because enteral feeding stabilizes gut barrier function, thereby reducing bacterial translocation, it is important early during the course of AP[14,105].

Therefore, whenever possible, *i.e.*, when dissipating pain allows the patient to eat and infectious parameters do not continue to rise, oral food intake should be initiated as early as possible[49]. If oral food intake is not possible and the patient needs nutritional support, enteral tube feeding is preferred over total parenteral nutrition. However, the composition of an optimal diet has not yet been evaluated.

Antibiotic prophylaxis: There also has been a change regarding prophylactic antibiotic therapy in patients with AP. While in the 90ties, prophylactic antibiotics where thought to improve the outcome in patients with AP, there is no emerging evidence that prophylactic antibiotics reduce infectious complications or mortality[106-108]. Today, there is no clear evidence that supports antibiotic prophylaxis as a routine treatment in patients with severe AP[109-111]. Prophylactic antibiotics may reduce pancreatic infection in special subgroups of patients, but further well-designed and adequately-powered studies are needed to definitively answer the clinical usefulness of antibiotic prophylaxis in these patients[108]. Therefore, antibiotic prophylaxis is currently not recommended by international guidelines for the treatment of acute pancreatitis[14,49].

CONCLUSION

Acute pancreatitis is a frequent and potentially life-threatening disease. Numerous clinical prognostic scoring systems have been developed, and yet tools to discriminate between mild, moderate, and severe AP early during the course of the disease are not well advanced. Therapy is currently mostly symptomatic with fluid resuscitation, pain management, and early oral feeding. However, most of these therapeutic approaches are not well-defined. Vigorous fluid resuscitation remains a cornerstone of early management of acute pancreatitis. Cross-sectional imaging during the early phase of evaluation has not been associated with improvement in outcome. There is no role for prophylactic antibiotics in the management of the early phase of AP. Enteral nutrition in AP can reduce mortality, systemic infections, and multiorgan dysfunction compared to parenteral nutrition. Immediate ERC is indicated only in patients with biliary pancreatitis with common bile duct obstruction

and cholangitis. These developments have contributed to an improved outcome for patients with acute pancreatitis, but further studies are still required to tackle the high mortality in this disease.

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2014 ADVANCES IN PANCREATITIS

Potential role of NADPH oxidase in pathogenesis of pancreatitis

Wei-Li Cao, Xiao-Hui Xiang, Kai Chen, Wei Xu, Shi-Hai Xia

Wei-Li Cao, Xiao-Hui Xiang, Kai Chen, Wei Xu, Shi-Hai Xia, Department of Hepatopancreatobiliary and Splenic Medicine, Affiliated Hospital, Logistics University of the Chinese People's Armed Police Forces, Tianjin 300162, China

Correspondence to: Shi-Hai Xia, MD, PhD, Department of Hepatopancreatobiliary and Splenic Medicine, Affiliated Hospital, Logistics University of the Chinese People's Armed Police Forces, 220 Chenglin Road, Hedong District, Tianjin 300162, China. xshhcx@sina.com
Telephone: +86-22-60578765 Fax: +86-22-24370605

Abstract

Studies have demonstrated that reactive oxygen species (ROS) are closely related to inflammatory disorders. Nicotinamide adenine dinucleotide phosphate oxidase (NOX), originally found in phagocytes, is the main source of ROS in nonphagocytic cells. Besides directly producing the detrimental highly reactive ROS to act on biomolecules (lipids, proteins, and nucleic acids), NOX can also activate multiple signal transduction pathways, which regulate cell growth, proliferation, differentiation and apoptosis by producing ROS. Recently, research on pancreatic NOX is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells, which are considered to be potentially associated with pancreatitis. In this review, we summarize the literature on NOX protein structure, activation, function and its role in the pathogenesis of pancreatitis.

Core tip: Besides directly producing the detrimental highly reactive reactive oxygen species (ROS) to act on biomolecules, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can also activate multiple signal transduction pathways, which regulate cell growth, proliferation, differentiation and apoptosis by producing ROS. Recently, research on pancreatic NADPH oxidase is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells, which are considered to be potentially associated with pancreatitis.

INTRODUCTION

Studies have demonstrated that reactive oxygen species (ROS) are involved in the pathogenesis of pancreatitis[1]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), a transmembrane flavoprotein enzyme, uses NADPH as an electron donor to catalyze the univalent reduction of oxygen, resulting in the production of superoxide free radical, which might be a source of oxi-

dants in injured pancreas[1]. NOX is mainly distributed in the phagocytic cell membrane with cytochrome C and *flavin adenine dinucleotide* groups, which can produce ROS, scavenging pathogenic microorganisms such as bacteria[2]. ROS, being generated by NOX, also participate in intracellular signaling processes in the pancreas. Recently, research on NOX is no longer limited to inflammatory cells, but extends to the aspect of pancreatic acinar cells and pancreatic stellate cells (PSCs) in pancreatitis patients[2]. The function of NOX, which is involved in the pathogenesis of inflammation in pancreatic acinar cells and PSCs, has become the hotspot of research. Non-phagocytic NOX derived ROS function as a messenger molecule to participate in the modulation of cell differentiation, proliferation and apoptosis in the pancreas. In this review, we summarize the literature on NOX protein structure, activation, function and its role in the pathogenesis of pancreatitis.

STRUCTURE, LOCATION AND FUNCTION OF NOX IN THE PANCREAS

NOX is a multicomponent enzyme consisting of five different subunits, including the subunits p22[phox] and gp-91[phox] (also known as NOX2) located in the membrane, together with the cytosolic subunits p40[phox], p47[phox] and p67[phox]. The participation of Rac would elicit full oxidase activity[3-5]. Relative to gp91[phox] (the catalytic subunit of NOX), p22[phox], p47[phox], p40[phox] and p67[phox] are regulatory subunits. Gp91[phox] in different types of cells has other six homologues, termed NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, which constitute the NOX family proteins[6-8]. NOX is an enzyme which was initially discovered in phagocytes[4,5]. NOX in neutrophils is composed of constitutive subunits (p22[phox] and gp-91[phox]) positioned in membrane and regulatory subunits (p47[phox] and p67[phox], and possibly p40[phox]) stationed in the cytosol[9]. In recent years, NOX has been discovered in several nonphagocytic cells such as fibroblasts[10], vascular smooth muscle cells[11] and hepatic stellate cells[12]. More recently, it has been found that NOX was present in pancreatic β cells[13,14], pancreatic acinar cells[15-18] and PSCs[19,20]. The main intrinsic components of NOX comprising the NOX2 isoform are present in human pancreatic islets[14]. Cytosolic subunits p47[phox] and p67[phox] as well as membrane-bound subunits p22[phox] and NOX1 are constitutively expressed in pancreatic acinar AR42J cells[16,21,22]. The key subunits of NOX including p22[phox], p47[phox], NOX activator 1 (a homologue of p67[phox]), NOX1, NOX4, and NOX2 (gp91[phox]) are expressed in PSCs[19,20]. The activation of non-phagocytic NOX is similar to that in neutrophils[23]. Upon activation of NOX, p47 translocates to the membrane and then recruits p67 to interact with the p22 subunit, thus facilitating NADPH-dependent formation of superoxide (O^{2-}), which increases the production of secondary ROS such as hydrogen peroxide (H_2O_2)[21]. Non-phagocytic NOX derived ROS function as a messenger molecule to participate

in the modulation of cell differentiation, proliferation and apoptosis[6-8]. NOX protein family can be activated quickly under pathophysiological conditions, leading to high production of ROS, which contributes to oxidative stress and a wide range of diseases.

ACTIVATION AND INHIBITION FACTORS OF NOX IN THE PATHOGENESIS OF PANCREATITIS

Cholecystokinin analogues

Cerulein, an analogue of cholecystokinin (CCK), can stimulate the pancreatic exocrine secretion by binding CCK receptors, causing the autolysis of pancreatic acinar cell[24]. There are two kinds of CCK receptor subtypes, CCK1 and CCK2 receptors. CCK1 receptors regulate pancreatic digestive enzymes, satiety and feeding behavior, while CCK2 receptors enhance the level of gastric acid, as well as gastrin which has anti-apoptotic effects on pancreatic cells[25]. Experimental pancreatitis induced with high dosages of cerulean, similar to human edematous pancreatitis, is characterized by cytoplasmic vacuolization, formation of edema and acinar cell death as well as elevation in serum levels of digestive enzymes caused by unconventional secretion of digestive enzymes[26]. ROS are involved in the activation of oxidant-sensitive nuclear transcription factor (NF-κB), expression of cytokine, apoptosis and further occurrence of pancreatitis[27]. P47[phox], p67[phox], NOX1 and p22[phox] in pancreatic AR42J cells could produce ROS after cerulein stimulation[21]. Intrapancreatic trypsin is not only activated by high-dose cerulein, but also regulated by neutrophils *via* NADPH oxidase[28]. The mechanism for the activation of NF-κB and expression of cytokines in pancreatic acinar cells stimulated by cerulein may be summarized as the following steps. Cerulein binds to the CCK receptor, a G-protein-coupled receptor, to activate phospholipase C (PLC) and inositol 1,4,5-trisphosphate (IP3), triggering transient Ca^{2+} release from the endoplasmic reticulum in pancreatic acinar cells. NOX activated by Ca^{2+} produces ROS to activate IκB kinase and then to phosphorylate IκB. Phosphorylated IκB can be ubiquitinated and degraded in a proteasome dependent manner to eliminate the inhibition of NF-κB, a p65/p50 heterodimer in the cytosol. NF-κB then translocates to the nucleus to mediate the expression of cytokines which are involved in the pathogenesis of pancreatitis (Figures 1 and 2)[27].

Renin-angiotensin system

The Renin-angiotensin system (RAS) is generally considered to regulate blood pressure and body fluid homeostasis[29]. The pancreatic RAS activation that is related to the production of ROS might contribute to oxidative stress and tissue injury[30,31]. Angiotensin Ⅱ, an active mediator of RAS, is transformed from angiotensin Ⅰ by the angiotensin-converting enzyme (ACE)[32]. The effect of angiotensin Ⅱ is regulated by its receptors, including
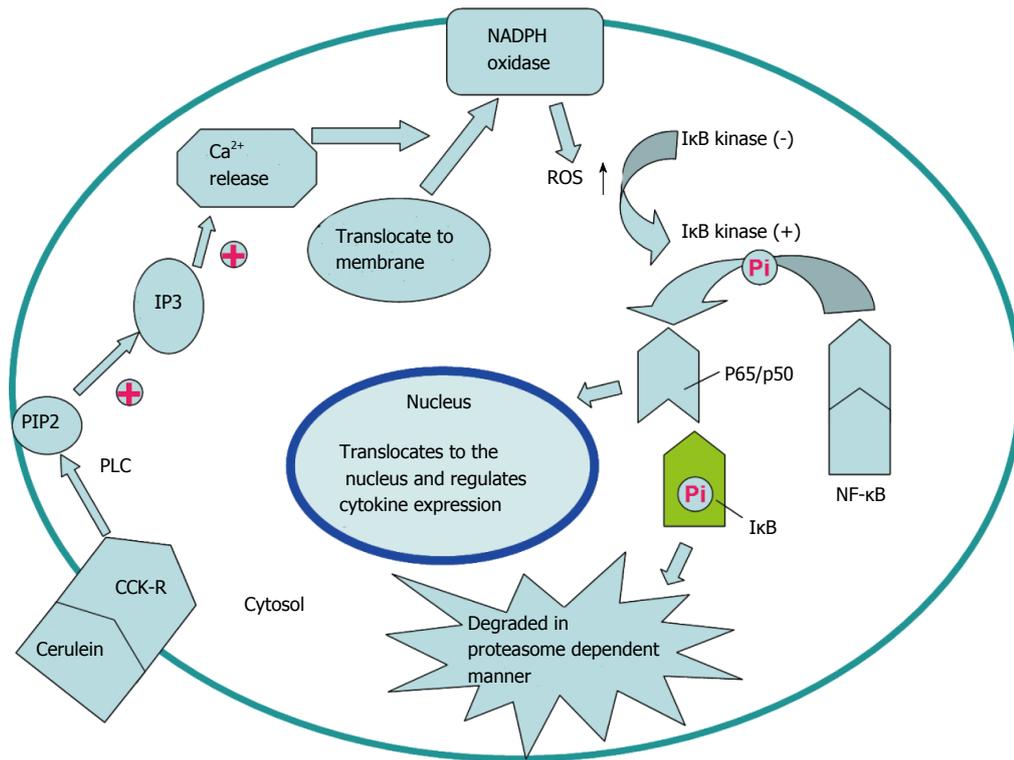
Figure 1 Potential mechanism of nicotinamide adenine dinucleotide phosphate oxidase activation *via* cholecystokinin receptor. Cerulein and cholecystokinin (CCK) receptor binding triggers transient Ca^{2+} release from the endoplasmic reticulum to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is mediated by PLC and IP3. Reactive oxygen species (ROS) generated by NADPH oxidase activate IκB kinase to phosphorylate IκB in the cytosol. Phosphorylated IκB is ubiquitinated and degraded in a proteasome-dependent manner. NF-κB translocates to the nucleus and regulates expression of cytokines to participate in the pathogenesis of pancreatitis.

angiotensin Ⅱ type 1 receptor (AT₁R) and angiotensin Ⅱ type 2 receptor (AT₂R)[32]. Many reports indicate that interaction of angiotensin Ⅱ with AT₁R promotes superoxide anion production through NOX system[30,31,33,34]. Inhibition of the AT₁R, but not AT₂R, may play a significant role in decreasing the severity of acute pancreatitis. Mechanism of NOX activation by AT₁R and AT₂R might contribute to different effects of AT₁R and AT₂R inhibitors on pancreatic injury induced by cerulein. Activation of pancreatic NOX was associated with oxidative stress which can be indicated by the level of protein oxidation in rats stimulated with cerulein[30,35]. However, further investigations about the potential application of RAS inhibitors including AT₁R in treating acute pancreatitis are needed in the future (Figure 2).

Ethanol and platelet derived growth factor

Alcohol abuse has long been recognized as the most common factor leading to chronic pancreatitis[36]. Activated stellate cells are viewed as vital regulators of chronic alcoholic pancreatitis or fibrosis. Hu *et al*[20] investigated the mechanisms of action of alcohol on PSCs to determine the correlation of NOX system and alcohol with the proliferation of PSCs. The results demonstrated that NOX activity was predominantly located in the cell membrane fraction (95%) compared to the cytosolic fraction (5%) of the stellate cells. platelet derived growth factor (PDGF) could increase NOX activity in a dose- and time-depen-

dent manner. PSC proliferation caused by alcohol is mediated by the activation of PDGF induced NADPH oxidase system. However, ethanol did not show a significant effect on stellate cell DNA synthesis, which provides a new perspective for the mechanism of fibrosis stimulated with alcohol (Figure 2)[20].

Vasoactive intestinal peptide

Previous reports found that vasoactive intestinal peptide (VIP) could decrease the production of cytokines to alleviate experimental acute pancreatitis[37]. VIP could decrease the level of ROS significantly and increase cell viability in acini cells in a dose dependent manner. NOX₁ and NOX₂ markedly increased following treatment with H_2O_2 in pancreatic acini. Besides, H_2O_2 can stimulate the activation of NOX. The production of ROS was affected by VIP *via* NADPH oxidase and the cAMP/PKA pathway because decreased NOX activity by administration of VIP could be abolished by PKA inhibitor H89. Oxidative stress and tissue injury in acini can be decreased by VIP through NOX inhibition (Figure 2)[38].

NOX SIGNAL TRANSDUCTION IN THE PATHOGENESIS OF PANCREATITIS

NOX protein family can be activated quickly under pathophysiological conditions, leading to high produc-
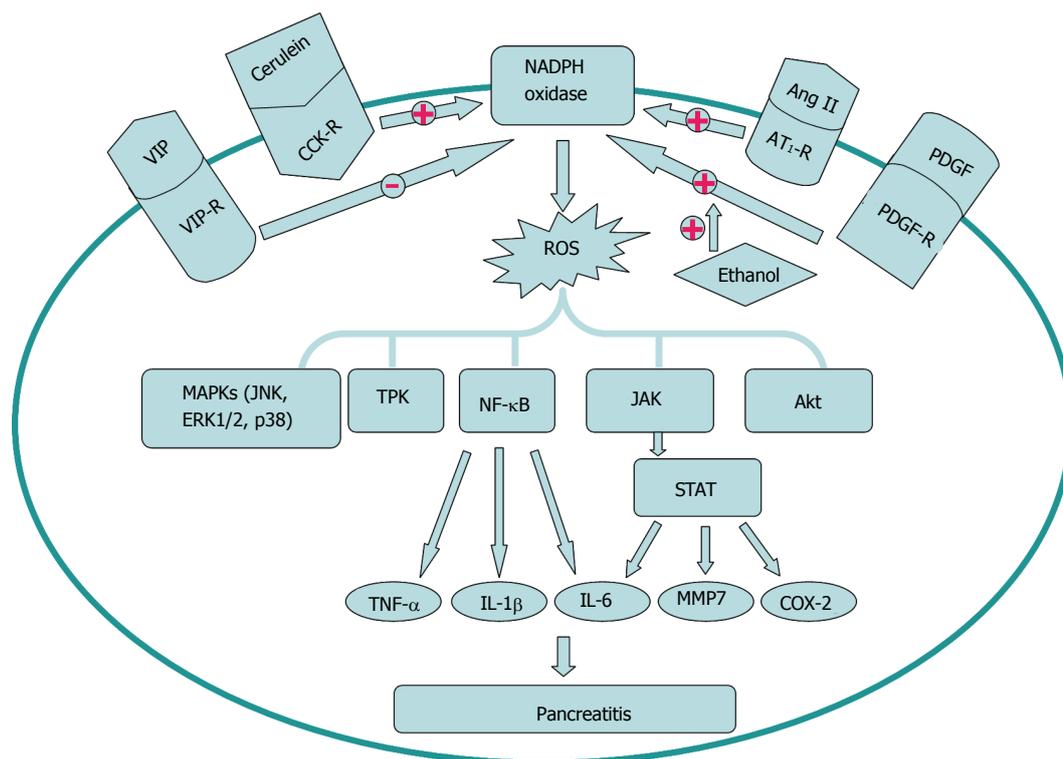
Figure 2 Activation and inhibition factors of nicotinamide adenine dinucleotide phosphate oxidase signal transduction in the pathogenesis of pancreatitis. Cerulein, Ang II and platelet derived growth factor (PDGF) can enhance, while VIP can decrease the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Ethanol can augment the activation of the cell's NADPH oxidase system stimulated by PDGF. The downstream signal molecules including MAPKs, TPK, NF-κB, JAK/STAT and Akt participate in the pathogenesis of pancreatitis. TNF: Tumor necrosis factor; IL: Interleukin; TPK: Tyrosine protein kinase; MAPK: Mitogen activated protein kinase.

tion of ROS, which contributes to oxidative stress and a wide range of diseases. Furthermore, ROS can act as an intracellular second messenger or chemoattractant to enhance the level of cytokines, resulting in the aggravation of pancreatitis[38]. Studies indicate that pro-inflammation cytokines such as IL-1β, IL-6 and TNF-α mediate the local or systemic manifestations of acute pancreatitis. IL-1β and TNF-α released from activated pancreatic macrophages respond to local tissue damage. Locally, these cytokines may aggravate the severity of acute pancreatitis. Systemically, IL-6 can increase the capillary permeability and accelerate the leukocyte adherence, leading to multiple organ failure (Figure 2)[27].

NF-κB and Janus kinase/signal transducers and activators of transcription

NF-κB, a member of the Rel family of transcription factors, can regulate the activation of cellular stress-related genes or early response genes such as growth factors, cytokines, adhesion molecules, and acute-phase proteins[39,40]. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway was relevant to the immune response mediated by numerous cytokines and non-immune response mediated by hormones and growth factors. The JAK/STAT pathway activated by the family of cytokine receptors regulate a variety of biological processes, such as immune response, cell survival, differentiation, proliferation and oncogenesis[41]. Recently, reports indicated

that cerulein could activate the JAK2/STAT3 pathway through NOX in pancreatic acinar cells[27].

NOX may be the source of ROS in pancreatic acinar cells during pancreatitis. ROS can induce expression of cytokines, apoptosis, NF-κB and JAK/STAT pathway activation, thus regulating the inflammation and apoptosis in pancreatic acinar cells. Consequently, NOX, NF-κB and JAK2/STAT3 may be involved in the pathogenesis of acute pancreatitis[27]. Inflammation and apoptosis in pancreatic acinar cells during pancreatitis may be alleviated by inhibition of NOX, NF-κB and JAK/STAT through suppression of inflammatory cytokines, apoptosis and caspase-3 activity. Ju *et al*[23] found that NOX inhibition suppresses STAT3-DNA binding, JAK2/STAT3 activation and TGF-β1 level in AR42J cells stimulated by cerulein. Therefore, ROS may activate NF-κB to induce cytokine production in pancreatic acinar cells through activation of NOX during pancreatitis[21]. NOX, NF-κB and JAK/STAT may be potential targets for treatment of acute pancreatitis.

Mitogen activated protein kinase and tyrosine protein kinase

Recently, studies found that mitogen activated protein kinase (MAPK) and tyrosine protein kinase (TPK) might be involved in NOX signal transduction pathway. ROS induced by the family of NOX can cause protein phosphorylation and cell apoptosis directly or indirectly.

In the direct way, ROS mediate the activation of the MAPK pathway and TPK pathway to promote protein phosphorylation in pancreatic acinar cells. ROS activate the signal transduction pathway which consists of different MAPK family members probably owing to the activation of the upstream ERK1/2 kinase pathway. ROS stimulate TPK signaling pathway through increasing the TPK activity, thereby promoting protein tyrosine phosphorylation and affecting signal transduction to regulate cell proliferation, differentiation, metabolism and apoptosis. Inhibition of NOX or ROS significantly reduced the p38MAPK signaling cascade[42]. Activation of the MAPK signaling pathway including SAPK/JNK, ERK1/2 and p38 by ROS induce cell apoptosis. The activation of the MAPK pathway is mainly dependent on the inhibition of tyrosine phosphatase by ROS[43].

In the indirect way, ROS reduce phosphatase activity, decrease protein dephosphorylation, and thus indirectly increase protein phosphorylation. ROS injure DNA, lipid and protein, thus indirectly inducing apoptosis. In some cases, NOX family can also inhibit cell apoptosis through ROS, which activate the pathway of NF-κB and Akt/ASK1, thereby reducing cell apoptosis[44].

NOX ACTIVATION IN DIFFERENT PANCREATIC CELLS INVOLVED IN THE PATHOGENESIS OF PANCREATITIS

Phagocytes

In support of the involvement of oxygen free radicals in acute pancreatitis, studies have addressed the possibility that the severity of pancreatitis can be reduced by inhibiting the activity of oxygen-derived free radicals[45]. ROS could have different origins, and the role of the NOX system in neutrophils but not pancreatic acinar tissue is originally considered essential. The phagocytic NOX is a multicomponent enzyme complex that is composed of membranous and cytosolic proteins in the resting cell. During activation, approximately 10% of cytosolic proteins including $p47^{phox}$ and $p67^{phox}$ are phosphorylated and translocate to the cell membrane to form active catalytic complexes with $p22^{phox}$ and $gp91^{phox}$, resulting in the generation of ROS[4]. Intrapancreatic trypsin activation and acinar cell trypsin-activation peptide (TAP) labeling induced by high dose cerulein were significantly decreased in neutrophil depleted rats. NOX deficient mice displayed attenuation of the cerulean-induced trypsin activation, while myeloperoxidase (MPO) deficient mice did not. Neutrophils have been considered to be implicated in pathologic activation of digestive enzymes by infiltrating the pancreas in acute pancreatitis, which is mediated by products of NOX[28].

Evidence suggests that inflammatory cell infiltration is an early and vital event in acute pancreatitis, which will lead to local and systemic complications[46]. Many of the pathological failures of acute pancreatitis may be a consequence of the overstimulation of leukocytes[47].

The argument put forward was that once pancreatitis has been initiated, chemoattractants for polymorphonuclear leukocytes, macrophages and platelets are released, possibly *via* the action of oxygen derived free radicals. The chemoattractants induce leukocytes and macrophages to adhere to the endothelium of the postcapillary venule and to migrate into the interstitial spaces. Stimulus-secretion coupling causes synthesis of a range of enzymes including elastase, cathepsins, phospholipase A_2, phospholipase C, platelet-activating factor (PAF) and MPO. When the quantity of material to be digested is excessive, phagocytosis may become so vigorous that the contents of leukocyte and macrophage granules are spilled outside the cell where they increase the severity of inflammation. As a result, large amounts of oxygen-derived free radicals are produced and may exceed the capacity of superoxide dismutase (SOD) and catalase to inactivate them[48].

Pancreatic acinar cells

ROS and apoptosis can be observed in pancreatic acinar cells in cerulein induced pancreatitis[49,50]. NADPH has been considered to be the major source of ROS in pancreatitis[18,21,22]. Oxidative stress induced inflammation and apoptosis have been implicated in pancreatitis[51,52]. Cerulein induced the expression of apoptosis-inducing factor (AIF). AIF is located in the mitochondrial membrane of pancreatic acinar cells. During apoptosis, AIF translocates from mitochondria to the cytoplasm and then enters into the nucleus, resulting in nuclear DNA aggregation and breakage to induce apoptosis of pancreatic acinar cells[53,54]. Antisense oligonucleotides (AS ODN) transfection or Ca^{2+} chelator treatment decreased the expression of AIF induced by cerulein in AR42J cells. These results suggested that intracellular Ca^{2+} increase and NOX activation might be the upstream events of AIF expression, which result in cerulein induced apoptosis of AR42J cells[18,55].

The activation of NOX was inhibited and the production of ROS was decreased when cerulein-stimulated pancreatic acinar cells were treated with Ca^{2+} chelator, which indicates that Ca^{2+} activate NOX and ROS. Transfection with AS ODN for NOX subunits $p22^{phox}$ and $p47^{phox}$ can inhibit the ROS generation, illustrating that NOX mediates the production of ROS. The apoptotic indices including apoptotic genes bax and p53, DNA fragmentation, caspase 3 activity, TUNEL staining and cell viability were inhibited by treatment with Ca^{2+} chelator or AS ODN transfection, indicating that NOX regulates ROS-induced apoptosis in a Ca^{2+} dependent manner in pancreatic acinar cells[22]. Diphenyleneiodonium (DPI), an inhibitor of NOX, reduces the AIF expression and caspase-3 activation, and thus inhibits apoptosis of AR42J cells[16]. During the stimulation with cerulein, the increase of NOX accelerates the formation of ROS in cells and mitochondria, thus further inducing the apoptosis of acinar cells[56,57]. ROS generated by pancreatic acinar cells stimulated with bile acids or cerulein can

induce apoptosis and, at the same time, induce pancreatitis[58-60].

Research indicates that JAK2/STAT3 activation and increases of MAPKs and TGF-β1 induced by administration of cerulein were inhibited by AS ODN transfection in AR42J cells, which shows that NOX can activate JAK2/STAT3, MAPKs and TGF-β1[23]. NOX may regulate the production of cytokines by activating NF-κB in AR42J cells stimulated with cerulein. Rebamipide, an antiulcer agent, can scavenge ROS and decrease the level of superoxide[61,62]. Transfection with AS ODN for NOX subunits or administration of DPI or rebamipide inhibited cerulein induced NF-κB activation and IL-6 expression[21]. Cerulein also could produce large amounts of ROS to activate NF-κB and thus stimulate the expression of cytokines in freshly isolated pancreatic acinar cells without inflammation[63].

Numerous studies have shown that increases of ROS and peroxidation products are accompanied with endogenous antioxidant depletion in the early stage of pancreatitis. Many preclinical antioxidant treatments, including genetic manipulation, significantly reduce pancreatic injury and inflammation[1,64-66]. However, randomized clinical trials of antioxidants have produced conflicting results[67], and treatment of pancreatitis with antioxidants has even been discontinued because of adverse events[68]. Moreover, several studies indicated that NOX was only present in neutrophils but not in pancreatic acinar cells[28,69].

PSCs

PSCs are the major fibrogenic cells in chronic injury of the pancreas, which encircle the acinus[70,71]. PSCs account for approximately 4% of the total pancreatic cells[72]. PSCs are quiescent in normal pancreas and can be identified by the character of vitamin A containing lipid droplets in the cytoplasm. When chronic pancreatitis happens, PSCs are activated and transformed into myofibroblast-like cells. As a result, intracellular lipid droplets disappear and α-smooth muscle actin (α-SMA) and extracellular components such as fibronectin and collagen arise[19,73]. Besides, PSCs may be involved in the pathogenesis of acute pancreatitis[72]. Therefore, suppression of PSC activation is a potential target to treat pancreatic inflammation and fibrosis.

Studies showed that p22[phox], p47[phox], NOX1, gp91[phox] (NOX2), and NOX4 were expressed in rat quiescent and culture-activated PSCs as well as human activated PSCs, while p67[phox] and NOX3 were not detected. NOX activator 1 was present in human PSCs, while NOX organizer 1 was not detected. NOX can activate PSCs, which can be verified by DPI inhibition experiments. Studies showed that DPI could inhibit the activation of PSCs, that is, to inhibit proliferation, chemokine production, α-SMA and collagen expression. Platelet-derived growth factor BB (PDGF-BB) promoted proliferation of rat PSCs, which was inhibited by DPI in a dose-dependent manner, showing that NOX underlies the PDGF in-

duced PSC proliferation. DPI decreased the chemokine production, which indicates that NOX also regulates the production of chemokines. DPI decreased the levels of α-SMA and collagen, once again, proving that NOX activate PSCs. DPI also inhibited interleukin 1β (IL-1β) and PDGF induced activation of MAPKs in PSCs, and this evidence indicates that NOX mediates the activation of MAPKs induced by IL-1β and PDGF in PSCs[19].

FUTURE RESEARCH ON THE PATHOGENESIS OF PANCREATITIS IN NOX

Accumulated evidence suggested that ROS induced by NOX play a significant role in pancreatitis. The activation of ROS mediates the activation of many cytokines[56,57]. ROS can induce cell apoptosis through direct and indirect pathways[43,44]. ROS induced by bile acids and cerulein can promote apoptosis of pancreatic acinar cells[18,69]. NOX is usually induced by cerulein, inflammatory factors, cytokines and growth factors as well as other stimuli in pancreatic acinar cells and PSCs. NOX can generate ROS, which in turn increase cytokines levels downstream to initiate the next activation cycle. The positive feedback of activation process might be one of the causes of pancreatitis. Although many scholars have made a great deal of research about the pathogenic mechanisms of NOX in the inflammation of pancreatic acinar cells and stellate cells, the relative importance of different pathogenic mechanisms of NOX in the pathogenesis of pancreatitis, the relationship between various pathogenic mechanisms of NOX, the specific pathways involved in each mechanism of NOX in pancreatitis, and the feasibility of NOX targeted therapy applied to pancreatitis are all needed to be studied in the future.

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Pancreatitis-imaging approach

Kiran K Busireddy, Mamdoh AlObaidy, Miguel Ramalho, Janaka Kalubowila, Liu Baodong, Ilaria Santagostino, Richard C Semelka

Kiran K Busireddy, Mamdoh AlObaidy, Miguel Ramalho, Janaka Kalubowila, Liu Baodong, Ilaria Santagostino, Richard C Semelka, Department of Radiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7510, United States

Author contributions: All authors contributed to this paper.

Correspondence to: Richard C Semelka, MD, Department of Radiology, University Of North Carolina at Chapel Hill, CB 7510-2001 Old Clinic Bldg., Chapel Hill, NC 27599-7510, United States. richsem@med.unc.edu

Telephone: +1-919-9669676 Fax: +1-919-8437147

Abstract

Pancreatitis is defined as the inflammation of the pancreas and considered the most common pancreatic disease in children and adults. Imaging plays a significant role in the diagnosis, severity assessment, recognition of complications and guiding therapeutic interventions. In the setting of pancreatitis, wider availability and good image quality make multi-detector contrast-enhanced computed tomography (MD-CECT) the most used imaging technique. However, magnetic resonance imaging (MRI) offers diagnostic capabilities similar to those of CT, with additional intrinsic advantages including lack of ionizing radiation and exquisite soft tissue characterization. This article reviews the proposed definitions of revised Atlanta classification for acute pancreatitis, illustrates a wide range of morphologic pancreatic parenchymal and associated peripancreatic changes for different types of acute pancreatitis. It also describes the spectrum of early and late chronic pancreatitis imaging findings and illustrates some of the less common types of chronic pancreatitis, with special emphasis on the role of CT and MRI.

Core tip: Imaging plays an important role in the diagnosis and staging of acute and chronic pancreatitis. Wider availability and good image quality makes computed tomography (CT) the mostly used imaging technique; however, magnetic resonance imaging (MRI) offers diagnostic capabilities similar to those of CT, with additional intrinsic advantages including lack of ionizing radiation and exquisite soft tissue characterization. This article reviews and illustrates the proposed definitions of the revised Atlanta classification for acute pancreatitis. It also describes the spectrum of early and late chronic pancreatitis imaging findings, with special emphasis on the role of CT and MRI.

INTRODUCTION

Pancreatitis is defined as the inflammation of the pancreas and considered the most common pancreatic disease in children and adults. It can be acute; representing an acute inflammatory process of the pancreas, or chronic; progressing slowly with continued, permanent inflammatory injury to the pancreas.

The incidence of acute pancreatitis is increasing in the United States and worldwide contributing to be one of the major sources of hospitalization. Acute pancreatitis was the most common gastrointestinal diagnosis for

hospitalization (with 274119 discharges) in the United States in 2009[1], usually running a mild clinical course[2]. However, a subset of patients develop severe disease independent of the degree of initial insult or etiology, with high morbidity and mortality up to 45%[3]. Over one-half of cases of acute pancreatitis in adults are related to cholelithiasis or alcohol consumption; whereas trauma, viral infections and systemic diseases account for the majority of cases in children[4].

The incidence of chronic pancreatitis is between five and twelve cases per 100000 persons per year; accounting for more than 120000 outpatient visits and 50000 hospitalizations annually[5]. Alcohol consumption accounts for the majority (80%) of cases of chronic pancreatitis in adults in developed countries; whereas malnutrition is the most common cause worldwide[4].

The purpose of our review is to illustrate the different imaging findings of pancreatitis on computed tomography (CT) and magnetic resonance imaging (MRI); with special emphasis on the revised terminology for acute pancreatitis and substantiate the increasing importance of imaging in the diagnosis, staging and follow-up of acute and chronic pancreatitis[5].

Acute pancreatitis
Acute pancreatitis results from the exudation of fluid containing activated proteolytic enzymes into the interstitium of the pancreas and leakage of this fluid into surrounding tissue.

There is general acceptance that a diagnosis of acute pancreatitis requires two of the following three features: (1) Sudden onset abdominal pain suggestive of acute pancreatitis (epigastric pain radiating to the back); (2) Serum amylase and/or lipase levels at least 3 times greater than the upper limit of normal; and (3) Characteristic imaging findings of acute pancreatitis on contrast-enhanced computerized tomography (CECT), MRI, or transabdominal ultrasonography (US) studies.

If abdominal pain is strongly suggestive of acute pancreatitis but the serum amylase and/or lipase activity is less than 3 times the upper limit of normal, characteristic findings on a CECT or MRI are required to confirm the diagnosis[6].

In order to assess and predict local or systemic effects of pancreatic injury, several disease severity-scoring systems were developed (*e.g.*, Ranson score, APACHE-II). In 1992, the Atlanta classification for acute pancreatitis was introduced to establish international standards of definitions of acute pancreatitis and its complications[7]. This system was designed to facilitate understanding and correlation of findings seen by gastroenterologists, pathologists, radiologists and surgeons; aiding improved communication between clinicians.

This initial Atlanta classification system represented major progress; however, advancing knowledge of the disease process, improved imaging and ever-changing treatment options warranted a revision, which was undertaken in 2012.

The revision of the Atlanta classification focuses heavily on morphologic criteria for defining the various manifestations of acute pancreatitis as outlined principally by means of CT and MRI.

Two distinct phases of acute pancreatitis were introduced: a first, or early, phase that occurs within the 1st wk of onset of disease; and a second, or late, phase that takes place after the 1st week of onset[7].

Early or first phase (less than 1 wk)
During this phase, pancreatic or peripancreatic ischemia or edema may completely resolve, develop fluid collections or progress to permanent necrosis and liquefaction. Severity of the acute pancreatitis in the early phase is entirely based on clinical parameters; mainly determined by the presence and duration of organ failure, but not the morphologic characteristics and its extent in and around the pancreas[8].

Late or second phase (after 1 wk from onset)
This phase occurs mostly in patients with moderate to severe acute pancreatitis and may extend for weeks to months. It is characterized by the presence of local complications, systemic manifestations (due to ongoing inflammation) and/or by transient or persistent organ failure. In this stage, the need for treatment is determined by presence of symptoms or complications, and the type of management is mainly based on the morphologic characteristics of pancreatic and peripancreatic region seen on cross sectional imaging. The severity of acute pancreatitis in late phase is determined by both morphologic criteria and clinical criteria like persistence of organ failure.

Updated terminology of acute pancreatitis
The web based international consensus[7] revised the original Atlanta classification of 1992 and proposed a new classification of acute pancreatitis to avoid the confusion in terminology seen over the last 2 decades. This consensus classification defines criteria for the diagnosis of acute pancreatitis (see above), differentiates the two types of acute pancreatitis (interstitial edematous pancreatitis and necrotizing pancreatitis) classifies the severity of acute pancreatitis into three categories and defines the morphology seen on imaging of pancreatic and peripancreatic collections that arise as complications of acute pancreatitis.

Role of imaging in acute pancreatitis
Imaging plays a significant role in the diagnosis of acute pancreatitis in clinically suspected cases or suggesting alternative diagnoses. It helps determine the causes of pancreatitis: gallstones, biliary duct obstruction or structural abnormalities. It also helps in grading the severity of the disease and identifying pancreatic or peripancreatic complications. Additionally, imaging can be utilized to guide therapeutic interventions.

The choice of appropriate imaging modality depends on the reason for investigation, clinical symptoms,

Table 1 Indications to perform contrast-enhanced computed tomography[58]

Types	Indications
Initial imaging	1 When the diagnosis of acute pancreatitis is uncertain
	2 Patients with hyperamylasemia, severe clinical pancreatitis, abdominal distention and tenderness, fever > 102°, and leukocytosis for the detection of complications
	3 Ranson score > 3 or APACHE score > 8
	4 Patients who fail to improve after 72 h of conservative medical therapy
	5 Acute change in clinical status, such as new fever, pain, and shock after successful initial medical therapy
Followup imaging	1 Acute change in clinical status suggesting complication
	2 7-10 d after presentation if CT severity score is 3-10 at presentation or grade
	3 To determine response to treatment after surgery or interventional radiologic procedures to document response to treatment.
	4 Before discharge of patients with severe acute pancreatitis

time of onset of symptoms and lab findings. However, CECT is the most commonly used modality in the evaluation of acute pancreatitis. In 2010, the ACR committee on appropriateness criteria and its expert panels have developed guidelines for determining the most appropriate imaging examinations for the diagnosis and treatment of acute pancreatitis and have given high score ratings[8,9] to CECT in different clinical scenarios. This is based on it is wide availability and high degree of accuracy. They also stated that MRI appears to offer diagnostic capabilities similar to multi-detector computed tomography (MDCT) with intrinsic advantages including the lack of ionizing radiation and the exquisite soft tissue characterization unmatched by any other imaging modality; allowing better depiction of stones and evaluation of the pancreaticobiliary ductal system.

Ultrasound

Ultrasound is frequently the first investigation performed on admission; although it has little value in the diagnosis of pancreatitis or its complications. Ultrasound is usually reserved to confirm or exclude the presence of stones or biliary dilatation. Early identification and treatment of these calculi may have a significant positive impact on outcome. However, body habitus of patient, operator dependence pose a limitation in detection of distal common bile duct stones accurately compared to CECT or MR imaging[9].

Ultrasound is limited in evaluating the entire pancreatic parenchyma; which is often partially or completely obscured by overlying bowel gas. It can however be helpful in monitoring the evolution of fluid collections, which occur as a result of acute pancreatitis, and in guiding diagnostic and therapeutic interventions.

CECT

CECT can show morphologic characteristic findings that allow for establishing the diagnosis of acute pancreatitis and determining the extent of disease severity. The best time for performing CECT in acute pancreatitis not well established and if performed immediately after the onset of symptoms, the full extent of pancreatic damage and its severity can be easily underestimated[10,11]. Conversely, a CECT obtained more than 5 d after onset of

symptoms that reveals a normal aspect of the pancreas or only mild inflammatory changes (fat stranding) surrounding the pancreas virtually excludes a severe form of acute pancreatitis[12].

Not all patients with acute pancreatitis need to undergo contrast-enhanced CT. In general, CT is not indicated in patients who are clinically classified as having mild pancreatitis (no clinical signs of severe pancreatitis) and show rapid improvement with appropriate medical management. CT should be used in patients who are classified as having severe pancreatitis or are at risk of developing severe pancreatitis; ideally after 72 h, to best assess the full extent of the disease[13].

CT should be repeated when the clinical picture drastically changes, such as with sudden onset of fever, decrease in hematocrit or sepsis. CT can also be useful to guide catheter placement for drainage and to assess success of treatment in patients who underwent percutaneous drainage or other interventions.

Furthermore, in patients with their first episode of pancreatitis who are over 40 years of age and have no identifiable cause for pancreatitis, contrast-enhanced CT should be used to exclude a possible neoplasm[13] (Table 1).

The main limiting factors for CECT are ionizing radiation, use of iodinated contrast material; especially in patients in with renal failure or contrast allergy and moderate sensitivity in identifying gallstones and biliary stones[14,15]. The above limiting factors can be overcome by using MRI; which does not use ionizing radiation; allowing it to be used during pregnancy, in patients with recurrent pancreatitis and for patients requiring multiple follow-up exams. Non-enhanced MRI seems to be more accurate and reliable for the early assessment of severity and prognosis of acute pancreatitis than is contrast-enhanced CT[16-18]; thus proving beneficial in patients with renal failure and history of contrast allergy.

MRI

Recent technological developments have dramatically improved the quality of abdominal MRI. Respiration, bowel peristalsis and vascular pulsations are major sources for artifacts affecting the accuracy and reproducibility of MRI. Breathing-independent sequences and respira-
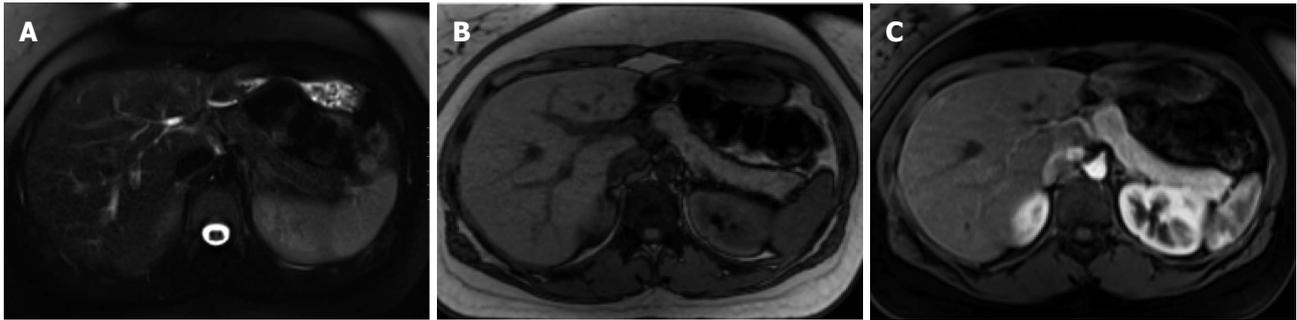
Figure 1 Normal pancreatic appearance on magnetic resonance imaging. A: Axial T2-weighted image with fat-suppression; B: Axial GRE out-of-phase T1-weighted image; C: Axial post-contrast 3D-GRE T1-weighted image with fat-suppression during the late arterial phase. The pancreas demonstrates low T2 signal intensity (A) and high T1 signal intensity on pre-contrast images (B), reflecting high protein content of the exocrine gland. The pancreas demonstrates avid homogenous enhancement on immediate post-contrast images (C), reflecting a normal capillary blush.
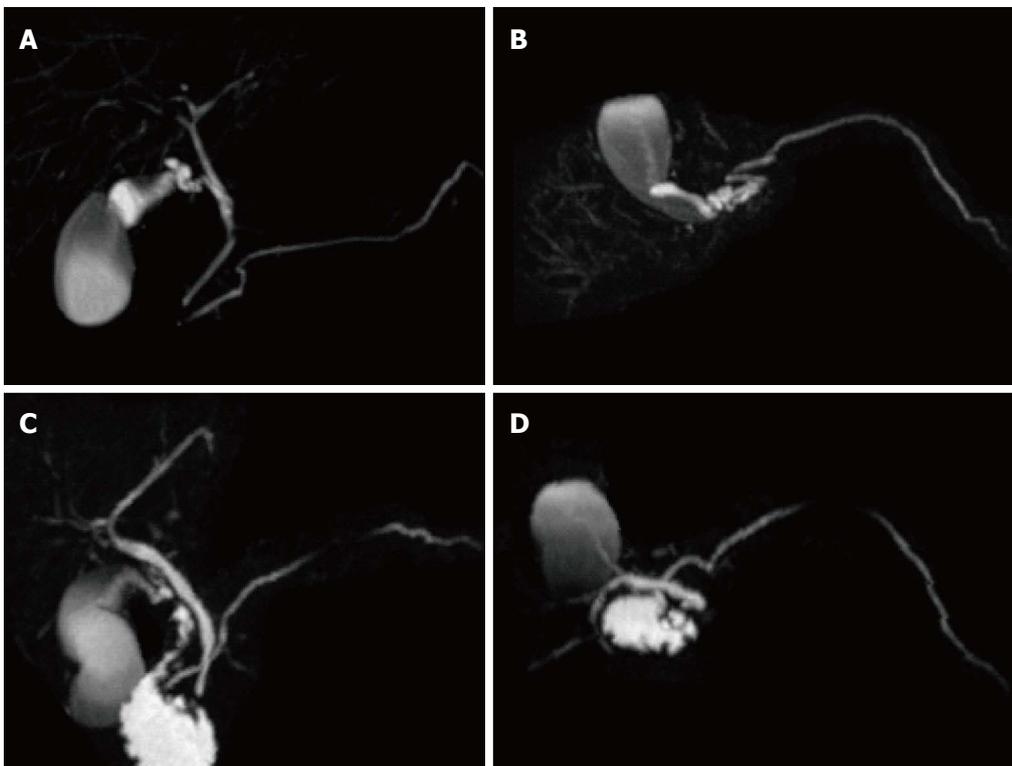
Figure 2 Normal pancreatic duct anatomy and pancreatic divisum. (A and C) Coronal and (B and D) axial post-processed maximum intensity projection 3D-MRCP images from two different patients. In the first patient, the main pancreatic duct courses inferiorly (A) and posteriorly (B), joins the CBD and opens in the major papilla in keeping with normal pancreatic duct anatomy. In the second patient, the main pancreatic duct continues its course superiorly (C) and anteriorly (D), crosses the CBD and opens in the minor papilla in keeping with pancreatic divisum.

tory gating techniques form the foundation of high-quality abdominopelvic MRI. New motion-robust MRI techniques provide promising results even in detection and characterization of pancreatic disease in patients that are not able to cooperate with breath-hold instructions[19].

A variety of pulse sequences are currently used for abdominal MRI including T1- and T2-weighted sequences with or without fat-suppression and post-gadolinium T1-weighted sequences (Figure 1). MR Cholangiopancreatography (MRCP) is routinely added to abdominal protocols to assess ductal obstruction, dilatation or course[20-22] (Figure 2); providing comprehensive evaluation of full range of pancreatic diseases. Due to the increasing incidence of acute pancreatitis due to gallstones in the United States, it is more beneficial to consider MRCP as an initial diagnostic study.

MRI is sensitive for detection of subtle changes of acute pancreatitis; particularly minor peripancreatic inflammatory changes; even in the setting of a morphologically normal pancreas on CT imaging; which may appear normal in up to 15%-30% of patients with clinical features of acute pancreatitis[23]. The sensitivity of MRI exceeds that of CT imaging, emphasizing its role in

Figure 3 Focal acute edematous pancreatic tail pancreatitis. A-C: Axial CT scan of the pancreas during the late arterial phase. There is evidence of ill-definition and reduced enhancement of the pancreatic tail (A and B), associated with mild peripancreatic fatty stranding extending to the anterior left perinephric space in keeping with focal acute edematous pancreatitis.

the evaluation of patients with clinically suspected acute pancreatitis and negative CT imaging findings.

It should be emphasized that MRI is a non-ionizing cross sectional imaging method and has a safer intravenous contrast profile in comparison to CT. This is particularly important in radiosensitive populations and those requiring repeated imaging follow up. Additionally, patients who present with acute pancreatitis often have a degree of renal impairment.

The factors that make CECT the most frequently applied imaging approach in pancreatitis are related to its universal availability (especially near the emergency room), faster scanning times, and relatively easier interpretability of CT images by physicians and general radiologists. For early presentation of acute pancreatitis, CT might be the preferred method for the reasons stated above. However, the adequate diagnostic performance of MRI along with the mentioned additive advantages favors MRI as the preferred method.

Endoscopic ultrasound

Endoscopic ultrasound (EUS) has shown great utility in providing high-resolution images of the pancreatic duct and parenchyma as well as extra hepatic biliary system; as the probe can be positioned in close proximity to the pancreas. Furthermore, EUS has become an invaluable technique for its ability to obtain targeted biopsies of lesions in and around the pancreas; thus, playing a prominent role in evaluating patients with atypical findings on other imaging studies.

The disadvantages of EUS are the requirement of monitored anesthesia care, need for expert endo-sonographer, modality operator dependence, and interobserver variability.

According to ACR appropriateness criteria, the role of endoscopic US in the evaluation of acute pancreatitis is primarily reserved for assessing and/or confirming choledocolithiasis and subsequent stone removal, as well as for identifying anatomic abnormalities (*e.g.*, pancreas divisum or malignancy) that can lead to acute pancreatitis. However, it has been recently proposed to use EUS in acute pancreatitis, as it is was found to contribute for the detection of causes like cancer, microlithiasis and chronic pancreatitis[24].

IMAGING-BASED MORPHOLOGIC TYPES OF ACUTE PANCREATITIS

Interstitial edematous pancreatitis

Interstitial edematous pancreatitis (IEP) is a milder form of acute pancreatitis that usually resolves over the first week. IEP is characterized by diffuse or localized enlargement of the pancreas secondary to interstitial or inflammatory edema without necrosis.

On CECT, findings include enlarged pancreas with relatively normal enhancement. Peripancreatic fat may be normal or show mild stranding and ground glass opacity due to inflammation, with small to varying amounts of non-enhancing peripancreatic fluid (Figure 3). The characteristic CECT finding that distinguishes IEP is absence of pancreatic parenchymal and peripancreatic necrosis.

On MRI, the signal intensity characteristics of the pancreas in IEP resemble those of normal pancreatic tissue. Enlargement of the pancreas, parenchymal edema and fat stranding are well demonstrated on T1-weighted images (Figure 4). T1-weighted imaging with fat suppression improves the delineation of the pancreas and pancreatic borders[25]. The pancreas demonstrates high signal intensity on pre-contrast fat suppressed T1-weighted images and enhances uniformly on immediate post-gadolinium images, reflecting a normal capillary blush. Fat suppressed T2-weighted sequences are very sensitive for detecting edema or minimal fluid and therefore have a role in detecting even milder forms of pancreatitis[26] (Figure 5).

Necrotizing pancreatitis

Necrotizing pancreatitis is the inflammation of the pancreas with obvious pancreatic and peripancreatic tissue necrosis. About 5%-10% of patients develop necrosis; affecting the pancreatic parenchyma in 5%, peripancreatic tissue in 20% and both in 70%. Pancreatic parenchymal necrosis carries a worse prognosis than peripancreatic necrosis[27].

Atlanta classification defines necrotizing pancreatitis as being associated with more than 30% parenchymal necrosis. The presence of less than 30% necrosis demands

Figure 4 Gallstone acute edematous pancreatic tail pancreatitis. A: Axial fast spin-echo (FSE) T2-weighted image with fat-suppression; B and C: Post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is mild diffuse lace-like increased T2 signal involving the pancreatic parenchyma, associated with a small amount of peripancreatic fluid near the pancreatic tail (A). The pancreas demonstrates diffuse minimal decrease in T1 signal intensity (B) and minimally reduced enhancement on the late arterial phase (C) in keeping with diffuse edematous pancreatitis. There are also innumerable gallstones (A).
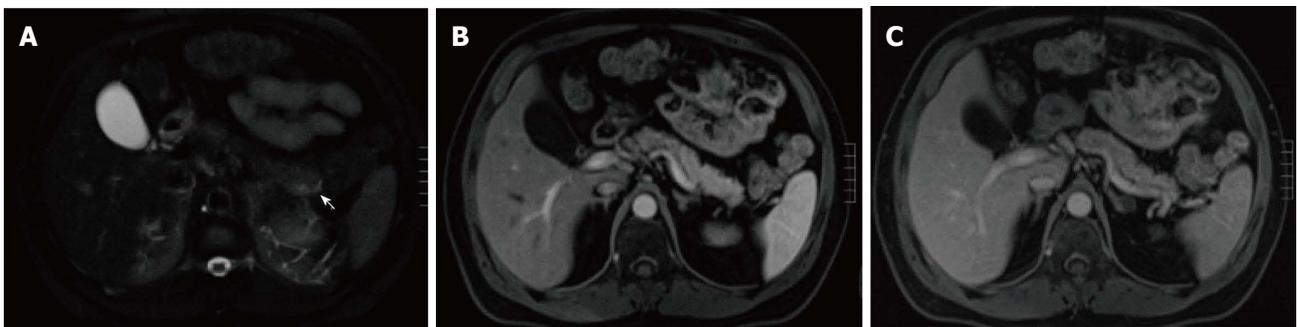
Figure 5 Subtle focal acute edematous pancreatic tail pancreatitis. A: Axial T2 weighted-image with fat-suppression; B and C: Axial post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is a very subtle area of increased T2 signal seen around the pancreatic tail (arrow, A), with fairly normal enhancement of the pancreas on the post-contrast images (B and C) in keeping with subtle focal acute edematous pancreatic tail pancreatitis.
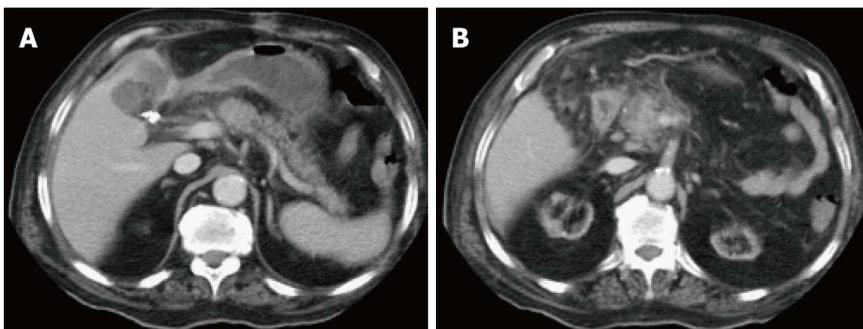

Figure 6 Focal pancreatic head necrotizing pancreatitis confined to the pancreatic parenchyma. A and B: Axial CT scan during the portal venous phase. There is evidence of significantly reduced enhancement of the pancreatic head (B), without peripancreatic extension or necrosis in keeping with focal acute necrotizing pancreatitis.

follow-up scanning in 1 wk to confirm true necrosis *vs* IEP[27].

On CECT, findings include areas of compromised pancreatic parenchymal enhancement on the post-Gadolinium images with or without peripancreatic inhomogeneous fluid collections (Figures 6 and 7). The impairment of pancreatic perfusion and signs of peripancreatic necrosis evolve over several days[28], which explains why an early CECT may underestimate the eventual extent of pancreatic and peripancreatic necrosis.

On MRI, necrosis shows appears as hypointense areas on T1-weighted images corresponding to areas of increased signal on fat-suppressed T2 weighted-images, associated with well defined areas of non-enhancing pancreatic parenchyma on post-Gadolinium sequences[29-31] (Figure 8).

For both CT and MRI, acquisition of an adequate arterial phase is of the utmost importance; as the maximum enhancement of pancreas is reached on the late arterial phase, and higher difference in signal between
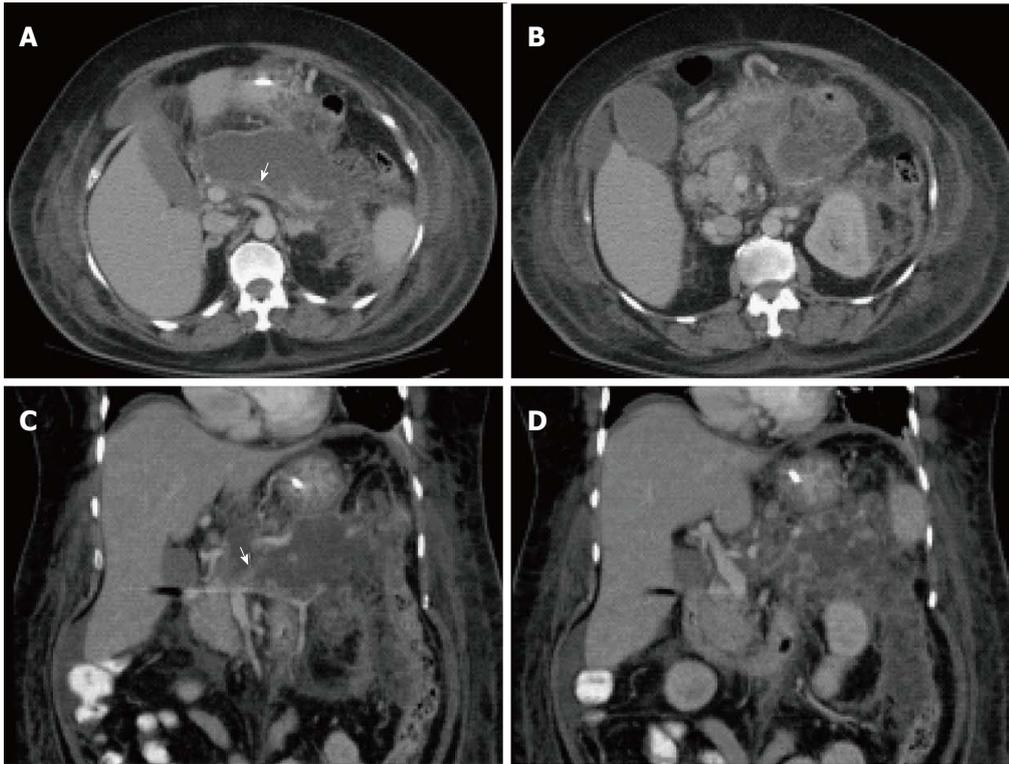
Figure 7 Severe acute necrotizing pancreatitis and peri pancreatitis. A-B: Axial CT scan during the late arterial phase; C-D: Coronal reformatted CT images. There is evidence of lack of arterial enhancement involving the pancreatic body and tail, which are replaced by necrotic tissue, associated with heterogenous peri-pancreatic tissue inflammation and necrosis extending to left perinephric space (A-B) and paracolic gutter (C-D), in keeping with severe necrotizing pancreatitis and peripancreatitis. There is also evidence of splenic vein thrombosis (arrow, A, C), a known complication of acute pancreatitis.
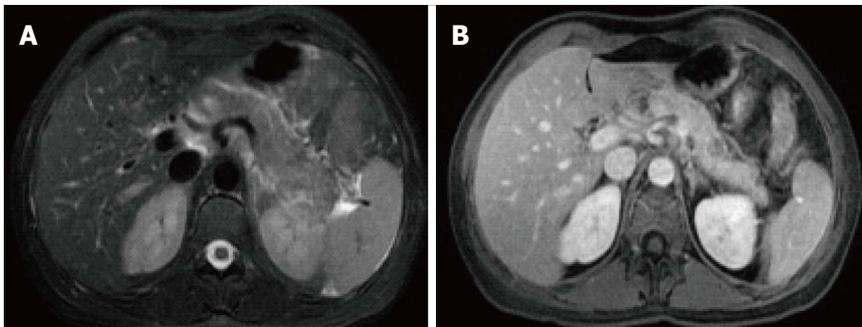

Figure 8 Focal acute necrotizing pancreatitis. A: Axial fast spin-echo T2- weighted image with fat-suppression; B: Axial post-contrast 3D-GRE T1- weighted images with fat-suppression during the venous phase. There is a focal area of low T2 signal involving the proximal part of the pancreatic tail, associated with minimal peripancreatic fat stranding (A). This focal area demonstrates significantly reduced enhancement on the post-contrast images, in keeping with focal necrotizing pancreatitis.

viable and necrotic is achieved on this phase.

Pancreatic duct disruption is an important prognostic factor. It is seen in 30% of the patients of necrotizing pancreatitis[32] when necrosis involves the central gland[33,34]. Drake *et al*[35] study showed that MRCP, a noninvasive imaging method, achieved 95% accuracy in detecting pancreatic duct disruption; thus helping in identifying patients who might benefit from early treatment.

Definition of pancreatic and peripancreatic collections

An important distinction is made between collections that are composed of fluid alone and those that arise from ne-

crosis and contain a solid component (and which may also contain varying amounts of fluid). Below, we define and illustrate the following terms: acute peripancreatic fluid collection; occurring in interstitial edematous pancreatitis, pancreatic pseudocyst as a delayed (usually after 4 wk) complication of interstitial edematous pancreatitis and necrosis; which may be an acute necrotic collection (in the early phase and before demarcation) or walled-off necrosis surrounded by an identifiable capsule on imaging (rarely develops before 4 wk).

Acute peripancreatic fluid collections

Fluid collections less than 4 wk in IEP lacking a discrete
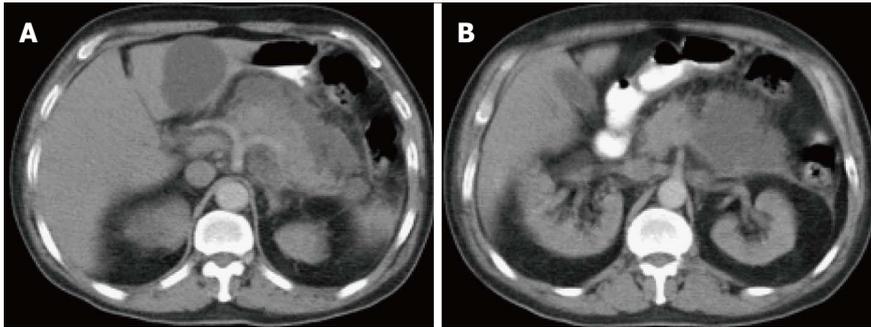
Figure 9 Acute interstitial edematous pancreatitis and acute peripancreatic fluid collections. A-B: Axial CT scan during the portal venous phase. The pancreas is mildly thickened and demonstrates mildly heterogenous enhancement, reflective of edema, in keeping with acute interstitial edematous pancreatitis. There is a peri-pancreatic fluid with imperceptible wall in keeping with acute peripancreatic fluid collections.
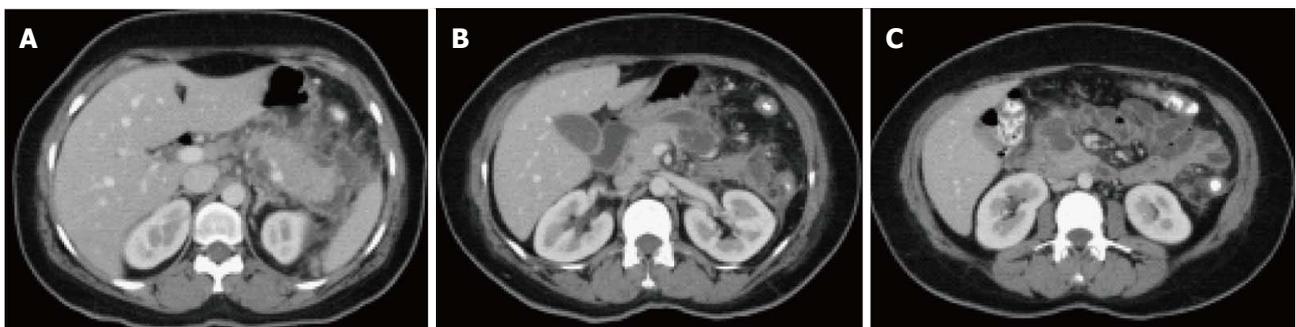

Figure 10 Peripancreatic fluid secondary to multifocal acute necrotizing pancreatitis. A-C: Axial CT images during the late arterial phase. There are two areas of focal necrosis involving the pancreatic body (B) and pancreatic head/uncinate process (C), associated with loculated peripancreatic fluid collection (A).
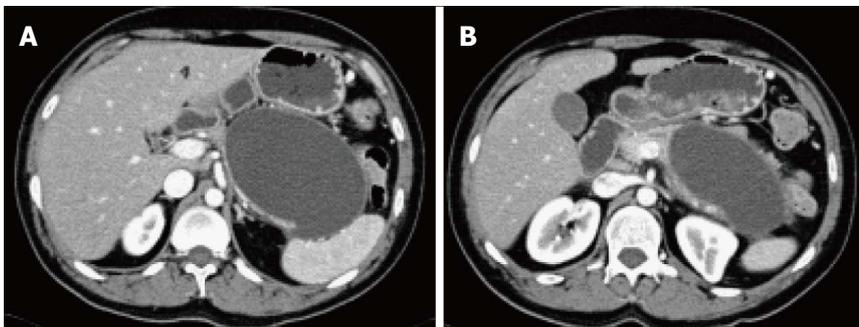

Figure 11 Large pancreatic pseudocyst. A-B: Axial CT scan during the late arterial phase. There is a large oval shaped pancreatic pseudocyst located anterior to the pancreatic body and tail, associated with mass effect on the thinned out pancreatic tissue in keeping with a large pancreatic pseudocyst.

wall, with no internal solid components in the peripancreatic region are called acute peripancreatic fluid collections (APFC). Approximately 50% of APFC's develop within 48 h following the onset of acute pancreatitis[36].

On CT scan, they appear as homogenous collections with low attenuation. They do not have well-defined walls and are confined by normal fascial planes in the retroperitoneum (Figure 9). They can be single or multiple (Figure 10). Most acute fluid collections remain sterile and usually resolve spontaneously without intervention[30].

On MRI, T2-weighted sequences are very sensitive in detecting peripancreatic fluid; which demonstrate high T2 signal intensity. On T1-weighted gradient echo images, APFC's demonstrate low signal intensity in a background of high signal intensity fat. No perceptible enhancement is depicted on post-gadolinium fat-suppressed T1-weighted images. The majority of fluid collections are typically confined to the lesser sac and anterior pararenal space or may track down to the pelvis and superiorly into mediastinum[29]. These collections are usually sterile and are spontaneously reabsorbed.

Pancreatic pseudocysts
Peripancreatic fluid collections that persist more than 4 wk in IEP, with a well-defined wall and no internal solid components in the peripancreatic region are called pseudocysts.
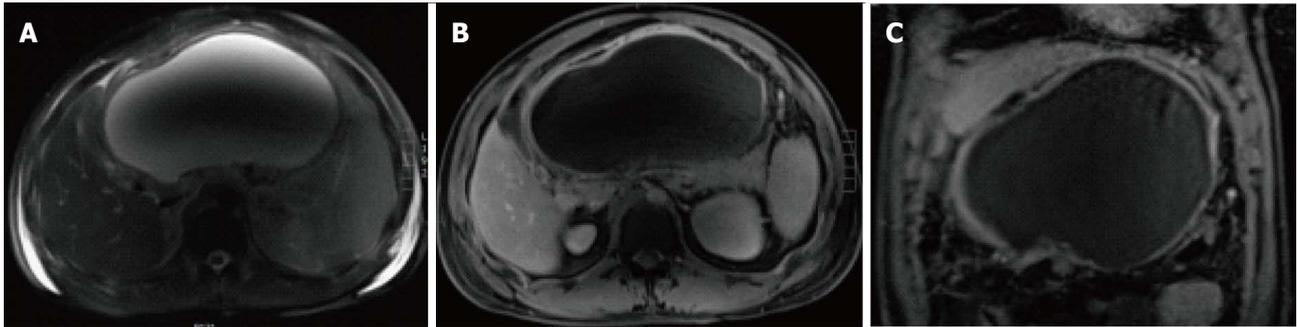
Figure 12 Large pancreatic pseudocyst. A: Axial fast spin-echo T2- weighted image with fat-suppression; B-C: Axial and coronal post-contrast 3D- GRE T1-weighted images with fat-suppression during the portal venous phase. There is a very large thin-walled cyst (A) within the lesser sac; which demonstrates mild uniform wall enhancement (B-C) in keeping with a large pancreatic pseudocyst. The central drop of signal on (A) is related to dielectric shading artifact.
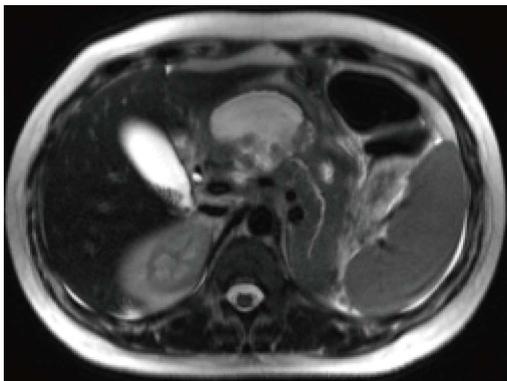
Figure 13 Acute necrotic collection. Axial T2 single-shot turbo spin-echo image. There is a well-defined fluid collection involving the pancreatic neck with peripancreatic extension and communication with the main pancreatic duct. This collection demonstrates well-defined outlines and heterogenous low T2 signal intensity debris within it in keeping with acute necrotic collection. Multiple gallstones are also noted.

On CECT, they appear as homogenous collections of low-attenuation surrounded by a uniform enhancing capsule (Figure 11). Typically, an increase of enhancement is observed in the interstitial phase; reflecting the presence of granulation tissue.

On MRI, pseudocysts demonstrate low in signal intensity on and T1-weighted gradient-echo images and relatively homogeneous high signal intensity on T2-weighted images. Pseudocysts walls enhance minimally on early post-gadolinium images and show progressively intense enhancement on 5-min post-gadolinium images; due to the presence of fibrous tissue (Figure 12).

Pseudocysts may sometime have communication with pancreatic duct and detecting this communication is helpful in the further patients' management. MRCP; a noninvasive imaging modality has an advantage of demonstrating possible communication between pancreatic pseudocyst and pancreatic duct.

The majority of pseudocysts resolve spontaneously. Infection and hemorrhage may complicate simple pseudocysts. Infected pseudocyst may contain gas bubbles on CT. However, absence of these findings on CT may further require confirmation by fine needle aspiration, when there is a strong clinical suspicion.

Acute necrotic collections

During the first 4 wk, a collection containing variable amounts of fluid and necrotic tissue is termed an acute necrotic collection (ANC). Unlike APFCs, ANCs are present within the pancreas and peripancreatic regions. ANC's may often maintain communication with the main pancreatic duct or one of its side-branches; for which, MRI can be useful in delineating this connection.

On CECT, ANC's demonstrate heterogeneous attenuation variably higher that of thin fluid (Figure 7). Follow-up imaging may be useful to characterize acute collections. CECT often shows ANC's as a homogenous non-enhancing area during the first week of necrotizing pancreatitis; making it difficult to be differentiated from APFC's. MRI may be helpful to confirm the presence of solid content in the collection.

On MRI, the necrotic debris may appear as irregularly shaped regions of low signal intensity within the necrotic collections. Breathing-independent T2-weighted sequences such as single-shot echo-train spin echo are useful to evaluate these necrotic collections (Figure 13); not only because of their high sensitivity in demonstrating the complexity of fluid, but also because many of these patients are very debilitated and are unable to co-operate with breath-holding instructions.

An advantage of MRI relative to MDCT in the evaluation of peripancreatic fluid collections is easier appreciation of solid debris with MRI[37]. The sensitivity and specificity of MRI in detecting solid debris of necrosis is 100% when compared to CT; which has a sensitivity of 25% and a specificity of 100%[38]. MRI can help in differentiating fluid collections secondary to pancreatitis from other cystic neoplasms.

Walled-off necrosis

After 4 wk, APFC's mature and develop thick non-epithelialized wall; acquiring the term walled-off necrosis (WON). They commonly occur in the pancreatic body and tail. Management for WON is different from pseudocyst as it contains non-liquefied debris; which needs to be surgically removed. Previously suggested nomenclature for this entity includes: organized pancreatic necrosis, pancreatic sequestration, pseudocyst associated

Figure 14 Necrotizing pancreatitis, with peripancreatic walled-off necrosis. A: Axial fast spin-echo T2-weighted image with fat-suppression; B-C: Axial post-contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and venous phase. There is a focal area of heterogeneous iso to slightly high T2 signal involving the pancreatic body-tail junction (A); which demonstrates lack of enhancement on the post-contrast images (B-C). There is associated sizable peripancreatic fluid collection; which demonstrates heterogeneous T2 signal intensity and thick enhancing wall post-contrast in keeping with walled-off necrosis.


Figure 15 Infected peripancreatic fluid in a patient with acute pancreatitis. A-C: Axial CT scan during the portal venous phase. There are a few gas bubbles seen within a small peripancreatic fluid (arrows, A-C). In the absence of any intervention in keeping with infected peripancreatic fluid.

with necrosis and subacute pancreatic necrosis.

On CECT, walled-off necrosis demonstrates a heterogeneous fluid and non-fluid attenuation with varying degree of loculations surrounded by a well-defined and enhancing encapsulating wall; which may involve both the pancreatic and extrapancreatic tissue. CECT, however, may not readily distinguish solid from fluid contents; as a result, pancreatic and peripancreatic necrosis may be misdiagnosed as a pancreatic pseudocyst. For this purpose, MRI may be required for this distinction (Figure 14).

Infected pancreatic necrosis

Pancreatic and peripancreatic necrosis can remain sterile or become infected. The development of secondary infection in pancreatic necrosis is associated with increased morbidity and mortality[3]. Most studies suggest that there is no absolute correlation between the extent of necrosis and the risk of infection and duration of symptoms[7]. The early diagnosis of infected pancreatic necrosis is very important in the initiation of antibiotic therapy.

The diagnosis of infected ANC or WON can be suspected in the presence of extraluminal gas on CT or MRI. This extraluminal gas is present in areas of necrosis and may or may not form a gas/fluid level depending on the amount of fluid content present at that stage of

the disease (Figure 15). The diagnosis may be confirmed by aspiration and analysis including microscopy and culture.

SEVERITY OF ACUTE PANCREATITIS

Clinical vs MCTSI vs MRSI severity index

Several clinical scoring systems like Marshal, SOFA. APACHE or Ranson criteria were designed to accurately correlate the complications like organ failure and mortality in acute pancreatitis. In the last two decades, radiological scoring systems were developed to accurately diagnose and correlate complications in acute pancreatitis.

For the first time in 1990, Balthazar *et al*[28] introduced the CT severity index for assessment of AP; which correlated well with morbidity, mortality and length of hospital stay. CTSI was widely adopted in clinical and research settings; however, a potential limitation was its inability to detect pancreatic necrosis. MCTSI introduced by Mortele *et al*[39] in 2004 to account for the limitations of CTSI (Table 2); which showed improved correlation with severity.

MCTSI incorporated extrapancreatic manifestations and simplified the evaluation of extent of parenchymal necrosis by categorizing into none, less than 30% or more than 30%; in addition to evaluating peripancreatic inflammation by detecting the presence or absence of

Table 2 MCTSI scoring ystem[39]

Prognostic Indicators	Characteristics	MCTSI[1]
Pancreatic inflammation	Normal pancreas	0
	Pancreatic ± peripancreatic inflammatory changes	2
	One or more collection or peripancreatic fat necrosis	4
Pancreatic necrosis	No necrosis	0
	< 30%	2
	> 30%	4
Extrapancreatic complications (pleural effusions, ascites, vascular, gastrointestinal, *etc.*)		2

[1]Scores ≥ 5 are associated with higher morbidity and mortality.

Table 3 MR severity index scoring system[69]

Prognostic Indicators	Characteristics	MRSI
Pancreatic inflammation	Normal pancreas	0
	Focal or diffuse enlargement of the pancreas	1
	Intrinsic pancreatic abnormalities with inflammatory changes in the peripancreatic fat	2
	Single, poorly defined fluid collection	3
	Two or more poorly defined collection or presence of gas in or adjacent to the pancreas	4
Pancreatic necrosis	No necrosis	0
	< 30%	2
	30%-50%	4
	> 50%	6

Table 4 Imaging criteria for chronic pancreatitis[70]

	CT criteria	MRI/S-MRCP criteria
Moderate chronic pancreatitis	≥ 2 of the following:	Moderate pancreatogram changes
	Main duct enlarged (2-4 mm)	Main duct abnormal and
	Slight gland enlargement (up to 2 × normal)	Abnormal side branches, > 3
	Heterogeneous parenchyma	
	Small cavities (< 10 mm)	
	Irregular ducts	
	Focal acute pancreatitis	
	Increased Density of the main pancreatic duct wall	
	Irregular head/body contour	
Marked chronic pancreatitis	with ≥ 1 of the following	Main duct abnormal and
	Large cavities (> 10 mm)	Abnormal side branches, > 3
	Gross gland enlargement (2 × normal)	Plus one or more of the following:
	Intraductal filling defects or pancreatic calculi	Large cavity
	Duct obstruction, stricture, or gross irregularity	Obstruction
	Contiguous organ invasion	Filling defects
		Severe dilatation or irregularity

peripancreatic fluid. Predictive accuracy of CT scoring systems for severity of AP and comparisons between CTSI and MCTSI were made[40]. They reported that they could not detect any significant differences between CTSI and MCTSI in evaluating the severity of AP. Their study also demonstrated that compared with APACHE II, both CT indexes more accurately diagnosed clinically severe disease and correlated better with the need for intervention and pancreatic infection.

It has been reported that MR severity index (MRSI) significantly correlated with CTSI (Table 3), Ranson score, C-reactive protein levels, appearance of systemic complications, duration of hospitalization and clinical outcome[17,41].

Chronic pancreatitis

Chronic pancreatitis is defined pathologically by continuous or relapsing inflammation of the organ leading to irreversible morphologic injury and typically leading to permanent impairment of both exocrine and endocrine functions. The incidence of chronic pancreatitis ranges from 5-12 per 100000 people in industrialized countries[1].

Chronic pancreatitis is a cause of abdominal pain, weight loss, steatorrhea and diabetes mellitus, which may occur as a consequence of multiple factors, including biliary stone disease, alcohol consumption, malignancy, metabolic disorders, and various genetic and environmental insults, including trauma[1].

The histopathological changes in chronic pancreatitis evolve from unevenly distributed fibrosis in early chronic pancreatitis to diffuse fibrosis involving the entire gland in late stages. In advanced disease, large areas of acinar parenchyma are replaced with sclerotic tissue causing atrophy. Ductal irregularities like strictures, dilatation and side branches ectasia occur due to surrounding fibrosis. Other characteristic findings of severe chronic pancreatitis are calcifications and presence of complications like pseudocyst, vascular aneurysms and venous thrombosis.

Role of imaging

Imaging plays a significant role in detecting parenchymal and ductal abnormalities in chronic pancreatitis and helps in differentiating early from advanced phases to a certain extent; which further guides the management of these patients.

Most commonly accepted CT- and MRI-based criteria for diagnosis of chronic pancreatitis are shown in Table 4.

Early chronic pancreatitis

Ultrasound and CT are insensitive in diagnosis of early chronic pancreatitis, as they often show no abnormalities. A recent study showed that parenchymal changes
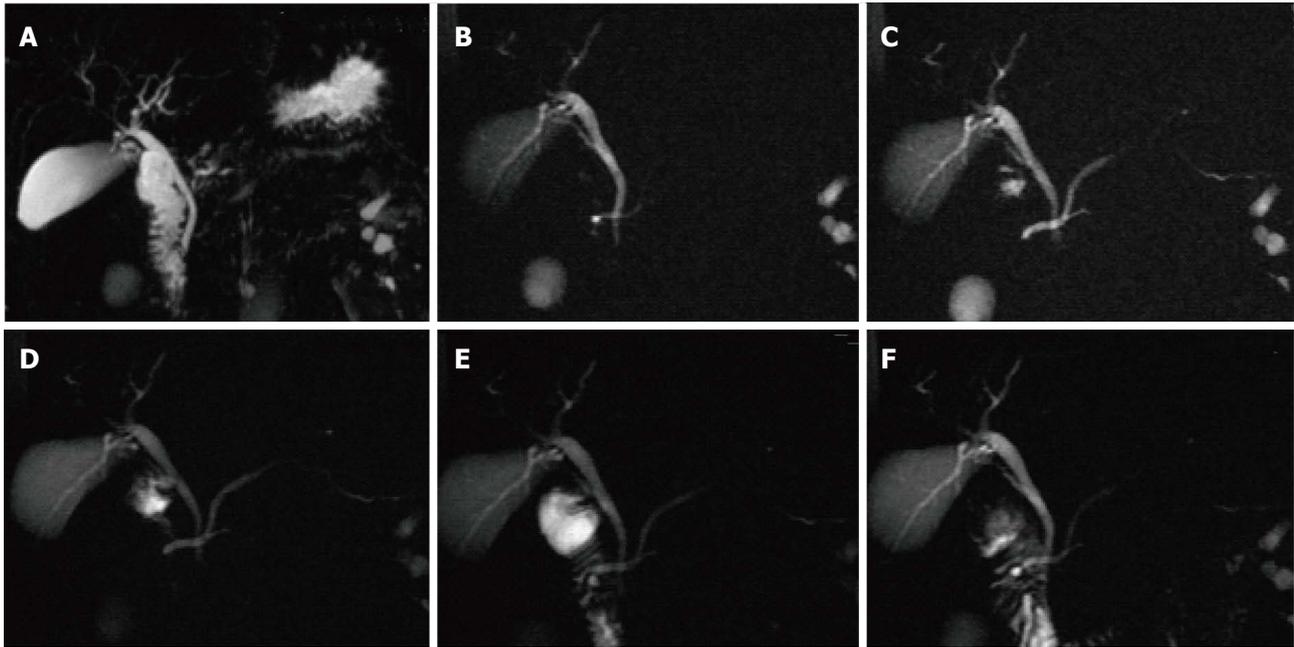
Figure 16 Pancreatic divisum, with a small Santorinicele. A: Coronal 3D- maximum intensity projection MRCP image before administration of secretin; B-F: Selected dynamic secretin thick-slab MRCP images obtained at 30 s (B), 60 s (C), 120 s (D), 4 min (E) and 9 min (F). Prior to administration of secretin, it is difficult to identify the main pancreatic duct (A). After administration of secretin, there is better delineation of the main pancreatic duct (C), with demonstration of pancreatic divisum. There is also enlargement of the accessory pancreatic duct, with demonstration of a small santorinicele (B, F). S-MRCP allows qualitative and quantitative assessment of pancreatic exocrine secretions. In this case, the pancreatic flow output was considered within normal limits; excluding early chronic pancreatitis.

might precede ductal changes in chronic pancreatitis; thus depicting the importance of MRI compared to MRCP in early diagnosis of disease[42].

On MRI, normal pancreas is hyperintense on T1 weighted images and shows uniform enhancement on the late arterial phase (Figure 1). MRI detects not only morphologic characteristics, but also early fibrotic changes. Fibrosis is shown by diminished signal intensity on T1-weighted fat-suppressed images and diminished enhancement on immediate post-Gadolinium gradient-echo images[43]. Low signal intensity on fat-suppressed T1-weighted images reflects loss of the aqueous protein in the acini of the pancreas. Diminished enhancement on capillary phase images reflects disruption of the normal capillary bed and increased chronic inflammation and fibrosis.

MRCP findings in early chronic pancreatitis often demonstrate normal main pancreatic duct with dilated and irregular side duct branches. The limiting factor is the underestimation of ductal size. Some investigators reported that patients with abnormal MR imaging findings but normal MRCP might benefit from dynamic secretin-MRCP (S-MRCP) (Figure 16); which may reveal ductal abnormalities due to improved visualization otherwise not detected on MRCP[42]. Secretin-MRCP has been reported to show ductal changes, like dilatations and strictures in early chronic pancreatitis.

EUS has a prominent role in chronic pancreatitis for its ability to detect early morphologic changes. Endoscopic retrograde cholangiopancreatography (ERCP) is considered to be gold standard test in detecting early

changes, but unlike ERCP, EUS is relatively a non-invasive procedure, and also helps in the evaluation of both pancreatic duct and parenchymal changes compared to ERCP that has limitation in evaluating pancreatic side branches and parenchyma[44]. Chong *et al*[45] showed sensitivity of 83% and specificity of 80% of EUS for the diagnosis of chronic pancreatitis.

Late chronic pancreatitis

CT is reported to be 60% to 95% sensitive in diagnosing advanced disease as it can readily detect parenchymal changes associated with advanced chronic pancreatitis[46]. Most common findings on CT include dilatation of main pancreatic duct and its side branches; which can be seen in 68% of patients. The ductal contour may be smooth, beaded or irregular[47].

Other findings include intraductal calcifications, which is the most specific finding and is seen in nearly half of the patients with chronic pancreatitis and parenchymal atrophy (Figure 17). However, parenchymal atrophy is neither specific nor sensitive as it seen normally with aging. Intraductal or parenchymal calcifications are usually seen with alcohol related chronic pancreatitis but not on chronic pancreatitis resultant from other causes.

All patients with late or advanced chronic pancreatitis show diminished signal intensity of the pancreas on T1-weighted fat-suppressed images, an abnormally low percentage of contrast enhancement on immediate post-contrast images, and progressive parenchymal enhancement on the 5-min delayed post-contrast images; reflecting the pattern of enhancement of fibrous tissue.
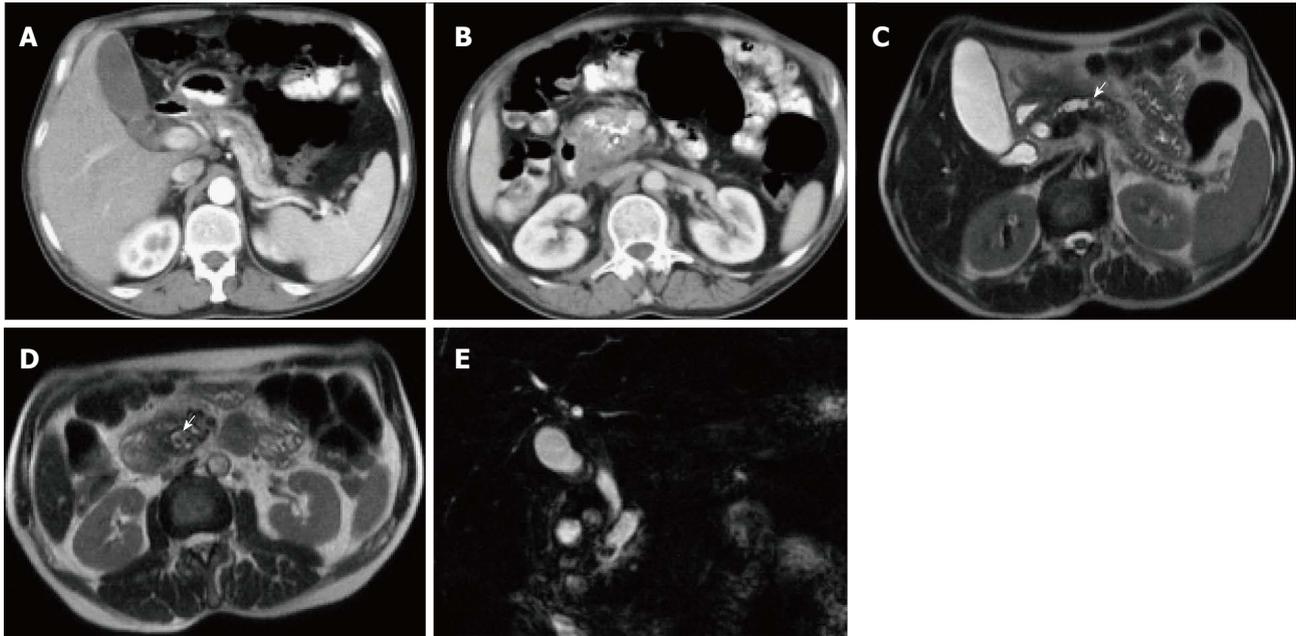
Figure 17 Chronic pancreatitis with pancreatic parenchymal calcifications and pancreatic duct stones. A, B: Axial CT scan during the late arterial phase; C, D: Axial T2 single-shot fast spin-echo images; E: Coronal 3D- Cholangiopancreatogram (MRCP) image. CT shows a markedly dilated and tortuous main pancreatic duct (MPD) (A, B), with foci of thick calcification involving the pancreatic head and uncinate process parenchyma (B). Large the proximal MPD stone was suspected on CT (arrow, B). MRCP shows gross pancreatic ductal dilatation with confirmation of the distal intraductal calculus (arrow, D), and shows an additional mid-pancreatic duct stone, not clearly seen on CT (arrow, C). No pancreatic masses or ductal anomalies are identified.
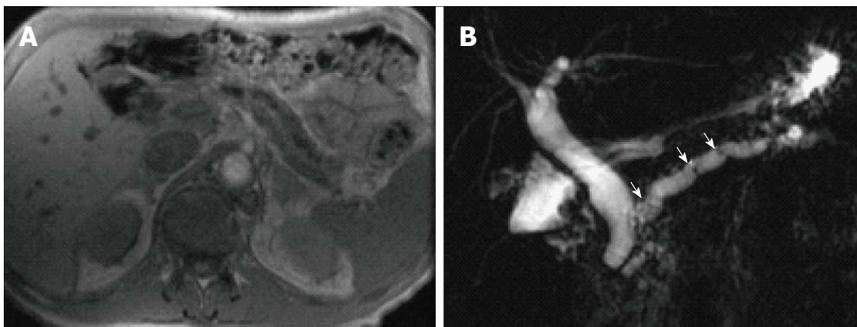

Figure 18 Chronic pancreatitis. A: Axial T1-weighted GRE MRI. B: Coronal-oblique thick-slab MRCP image. There is evidence of diffuse thinning of the pancreatic parenchyma with uniform dilatation of the pancreatic duct and prominence of the pancreatic duct side-branches (A-B), associated with multiple tiny stones at the proximal pancreatic duct (arrows, B) in keeping with chronic pancreatitis. There is also mild uniform dilatation of the CBD, which tapers down to the level of the pancreatic duct (B).

MRCP in advanced phase demonstrates dilatation of the main pancreatic duct with ectasia of the side branches (Figure 18); giving chain of lakes appearance manifested as pancreatic ductal strictures, irregularities and intraductal calculi, appearing as hypointense filling defects.

Enlarged pancreatic head in chronic pancreatitis vs adenocarcinoma

Chronic pancreatitis may involve only the pancreatic head in 30% of patients, resulting in focally enlarged pancreatic head. In these cases, the focus of chronic pancreatitis can simulate the appearance of pancreatic ductal adenocarcinoma.

Both chronic pancreatitis and adenocarcinoma show similar imaging characteristics on CT and MRI due to abundant fibrosis and ductal obstruction; therefore, making the differentiation between these two entities very difficult. Both are generally seen as hypodense lesions on CT, mildly hypointense on T1-weighted images and heterogeneously mildly hyperintense signal on T2-weighted images. However, certain imaging characteristics are helpful in distinguishing enlarged pancreatic head in chronic pancreatitis from adenocarcinoma (Table 5).

Rarely, chronic pancreatitis may involve only the focally enlarged portion of the pancreas, with the reminder of the pancreas having no inflammatory changes. In these cases, the focus of chronic pancreatitis can also simulate the appearance of pancreatic ductal adenocarcinoma. The inflammatory process may also be sufficiently

Table 5 Differentiating imaging features between chronic pancreatitis and pancreatic adenocarcinoma

Chronic pancreatitis	Pancreatic adenocarcinoma
Preserved glandular, feathery or marbled texture similar to that of the remaining pancreas	Definable, circumscribed mass lesion is most often diagnostic for tumor, which disrupts the underlying architecture and results in loss of anatomic detail
Heterogeneous pancreatic enhancement with presence of signal void (cysts and calcifications) on immediate post-gadolinium images	Irregular, heterogeneous, diminished enhancement on postgadolinium images compared to adjacent pancreatic parenchyma
Irregular dilatation of main pancreatic duct with gradual narrowing	Abrupt cut off of the pancreatic duct with significant proximal dilatation +/- presence of double duct sign
Presence of multiple intraductal calcifications (the most specific finding)	Very few ductal calculi compared to chronic pancreatitis
Dilatation of main pancreatic duct with and ectasia of the side branches, giving chain of lakes appearance	Minimal dilatation of side branches
No vascular encasement, significant lymphadenopathy or distant metastasis	Vascular encasement, lymphadenopathy or distant metastasis

destructive that underlying stromal pattern is lost. In these rare cases, diagnosis can only be established by surgical resection and histopathological examination to confirm the absence of malignancy.

Despite the high-resolution images produced by conventional EUS, there are no specific EUS imaging features that can differentiate pancreatic cancer from other common mimics, including lymphoma, focal pancreatitis, neuroendocrine tumors, metastases, and focal AIP[48]. However, one of the strengths of EUS is its ability to allow guided fine needle aspiration (FNA); which may overcome this problem.

In a retrospective analysis by Agarwal *et al*[49], 110 patients with abnormal CT or MRI with an enlarged head of the pancreas or dilated pancreatic duct with or without dilation of the common bile duct underwent EUS or EUS-FNA. The study revealed an accuracy of 99.1% for EUS and/or EUS-FNA in diagnosing pancreatic neoplasm with a sensitivity of 88.8% and specificity of 100%[49]. Given the high accuracy in the evaluation of pancreatic tumors, Eloubeidi *et al*[50] proposed routine EUS-FNA for the differential diagnosis of solid pancreatic masses. Other studies have shown that a negative EUS in ambiguous cases (where a mass is suspected) has a high negative predictive value[51,52].

Positron emission tomography-computed tomography (PET-CT) has an established role in the diagnosis of pancreatic carcinoma, especially when cross sectional imaging or biopsies are equivocal or nondiagnostic. In patients with a suspicion of pancreatic malignancy, a focal increase in ^{18}F-fluorodeoxyglucose (FDG) uptake suggests the diagnosis of malignancy. Nonetheless, the cutoff value of maximum standardized uptake value (SUVmax) is not defined, as it overlaps in benign and malignant pancreatic disease processes[53,54].

Furthermore, FDG-PET's detectability of pancreatic cancer depends on lesion size and degree of FDG uptake and surrounding background uptake. In the setting of chronic pancreatitis, FDG-PET is shown to detect pancreatic adenocarcinoma with a sensitivity of 92% and with a negative predictive value of 87%. In the set-

ting of acute pancreatitis, the specificity can be as low as 50%, as it is known that inflammatory tissue can also demonstrate FDG activity[47].

Complications of chronic pancreatitis
The most common non-neoplastic complications of chronic pancreatitis include pseudocysts, pseudoaneurysms (due to erosion of the arterial wall), splenic vein thrombosis with subsequent development of collaterals, biliary obstruction (due to pseudocysts), and gastrointestinal complications like gastric outlet obstruction or bowel ischemia[6,55]. These complications are well depicted with CT and MRI.

MRI with MRCP may be superior to CT in detecting specific complications like pseudocysts, fistula formation, distal common biliary dilatation and vascular complications associated with higher morbidity and mortality[46].

Special types of chronic pancreatitis autoimmune pancreatitis.

Autoimmune pancreatitis is a distinct form of pancreatitis characterized clinically by obstructive jaundice (with or without pancreatic mass), histologically by a lymphoplasmacytic infiltrate and fibrosis and therapeutically by a dramatic response to steroids[56].

Autoimmune pancreatitis accounts for 2%-6% of chronic pancreatitis[57,58]. It is associated with other autoimmune disorders like Sjogren's syndrome, primary biliary cirrhosis, and primary sclerosing cholangitis[59,60]. Early diagnosis of autoimmune pancreatitis is crucial as it often responds to steroid therapy; thus avoiding complications.

In AIP, affected areas appear enlarged and hypodense on CT. CECT demonstrates diminished enhancement of the involved parenchyma on the late arterial phase and delayed enhancement on the delayed phase (Figure 19). The MR appearance of autoimmune pancreatitis is similar and is characterized by enlarged pancreas with moderately decreased signal intensity on T1-weighted images, mildly high signal intensity on T2-weighted images and delayed post-gadolinium enhancement of the pancreatic
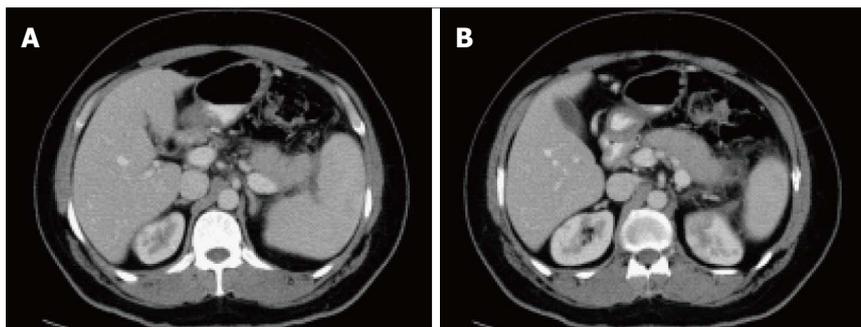
Figure 19 Autoimmune pancreatitis. A, B: Axial CT scan during the late arterial phase. There is evidence of diffuse pancreatic swelling with loss of the normal pancreatic lobulation, obliteration of the pancreatic duct and subtle low attenuating peripancreatic rim (A, B) in keeping with autoimmune pancreatitis. Patient had high IgG4 level (> 0.500 g/L).
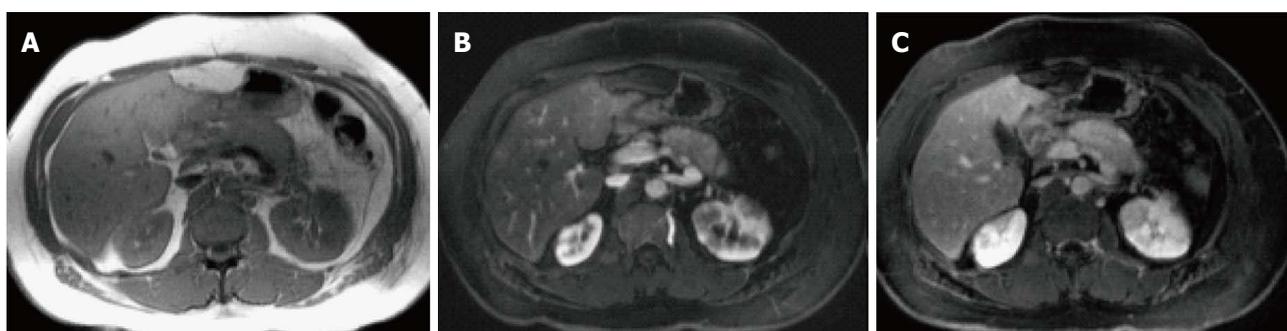
Figure 20 Autoimmune pancreatitis. A: GRE T1-weighted image; B, C: Post- contrast 3D-GRE T1-weighted images with fat-suppression during the late arterial and portal venous phases. There is evidence of diffuse pancreatic swelling with reduced T1 signal, loss of the normal pancreatic lobulation and obliteration of the pancreatic duct, associated with a rim of low T1 signal (A). The pancreas demonstrates diffuse reduced enhancement on the late arterial phase and progression of enhancement on the portal venous phase in keeping with autoimmune pancreatitis. The patient had significant biliary tree irregularities in keeping with primary sclerosing cholangitis (not shown). Additionally, there are a few bilateral wedge-shaped areas of renal hypo- enhancement in keeping with segmental infarcts.

parenchyma (Figure 20). Additional findings that may be observed in autoimmune pancreatitis include: (1) capsule like rim surrounding the diseased parenchyma, that is hypointense on T2-weighted images and may show delayed post-gadolinium enhancement[59]; (2) absence of parenchymal atrophy; (3) ductal dilatation proximal to the site of stenosis; (4) absence of peripancreatic fluid; and (5) clear demarcation of the abnormality[60].

MRCP depicts diffuse or segmental narrowing and irregularity of the main pancreatic duct as characteristic findings. The most commonly involved segment is the intrapancreatic common bile duct, and less frequently multifocal intrahepatic biliary strictures are noted.

Autoimmune pancreatitis is has 3 types based on morphologic patterns: diffuse, focal, and multifocal. Diffuse disease is the most common type. CT and MRI commonly show a swollen, sausage-like pancreas with poorly demonstrated borders and a capsule-like rim of low-density/intensity[61].

The diffuse form of AIP may mimic diffuse disorders like lymphoma, metastases or other diffuse infiltrative processes. In most of these disorders, unlike AIP, the parenchyma is heterogeneous and shows irregular contours.

Focal disease is less common and manifests as a well-defined hypodense mass, often involving the head and mimicking pancreatic adenocarcinoma. In patients who underwent pancreatic resection for suspected malignancy, 2.5%-8% were ultimately diagnosed with AIP without malignancy[58,62]. However, the probability of AIP *vs* pancreatic cancer in patients with obstructive jaundice can be predicted based on CT/MRI findings.

Diffusely enlarged pancreas showing low density mass with enhancement on delayed phases on CT/MRI, especially with a capsule-like rim, and no pancreatic ductal cutoff is highly likely to have AIP. Low-density mass on CECT, pancreatic ductal cutoff in presence or absence of pancreatic atrophy mostly suggests pancreatic cancer.

Groove/paraduodenal pancreatitis

Groove pancreatitis is a rare form of focal chronic pancreatitis involving the anatomic groove between the pancreatic head, duodenum and common bile duct. Groove pancreatitis is categorized into 2 forms: pure, involving exclusively the groove; and segmental, involving the groove and extending in to the pancreatic head[63] (Figure 21).

Pathogenesis remains controversial but may result from obstruction of the accessory pancreatic duct as it drains into the second portion of the duodenum through the
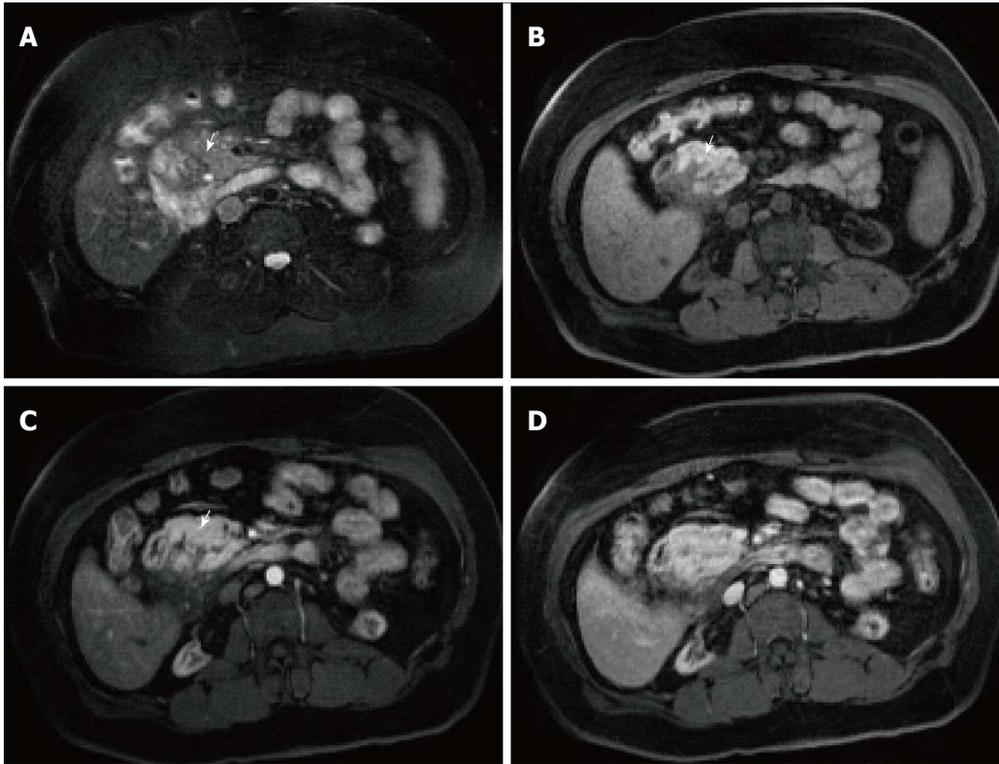
Figure 21 Groove pancreatitis. A: Axial T2-weighted single-shot fast spin-echo (SS-FSE) images with fat-suppression; (B) Pre- and (C, D) Post-contrast 3D-GRE T1- weighted images with fat-suppression during the late arterial and portal venous phases. There is a slightly low T2 signal sheet-like mass in the pancreaticoduodenal groove, with tiny cystic changes (arrow, A). The mass shows low T1 signal with extension into the pancreatic head (arrow, B). Imperceptible enhancement is depicted on the immediate post-contrast image (arrow, C), with progressive enhancement on the subsequent delayed images (D) in keeping with groove pancreatitis.

minor ampulla[64]. Presence of cystic changes, frequently located in the expected region of the pancreatic accessory duct, is considered a prominent feature of this process, likely related to accessory duct obstruction[65]. It is commonly seen in patients with history of alcohol abuse[64].

The classic MDCT features in the pure form can range from ill-defined fat stranding to frank soft tissue within the pancreaticoduodenal groove with increased delayed enhancement due to fibrosis. Thickening of medial duodenal wall on coronal images and presence of cysts can be appreciated sometimes[66]. On MRI, groove pancreatitis is characterized by a sheet-like mass in the groove that shows low signal on T1-weighted images, slightly high signal on T2-weighted images relative to the pancreas and may show delayed enhancement. Cystic lesions are well shown on T2-weighted images in the groove or duodenal wall[63].

It may be challenging to differentiate groove pancreatitis from pancreatic head duct adenocarcinoma. Recently, it was shown that by using three strict diagnostic criteria for groove pancreatitis: (1) focal thickening of the second portion of the duodenum; (2) abnormal increased enhancement of the second portion of the duodenum; and (3) cystic changes in the region of the pancreatic accessory duct, distinction from pancreatic duct adenocarcinoma could be achieved with high diagnostic accuracy (87.2% of patients), and a diagnosis of cancer could be excluded with a negative predictive value of 92.9%[67].

Hereditary pancreatitis
Hereditary pancreatitis is an autosomal dominant disease

presenting as multiple episodes of pancreatitis in the absence of any predisposing factors. Imaging findings include parenchymal and intraductal calcifications and parenchymal atrophy. However, in hereditary pancreatitis, imaging plays an important role to rule out structural causes of pancreatitis and to closely monitor the development of pancreatic cancer, the risk of which is increased by many folds in these patients.

CONCLUSION

In summary, imaging plays an important role in the diagnosis and staging of acute and chronic pancreatitis. Both CT and MRI are widely used and represent the best cross sectional techniques in the setting of pancreatitis. Wider availability and good image quality make CT the mostly used imaging technique; however, due to its nonionizing nature, unmatched soft tissue contrast and higher safety profile of intravascular contrast media make MRI particularly valuable in pregnant patients, patients with recurrent pancreatitis and patients requiring multiple follow up examinations. Also, early form of chronic pancreatitis and some specific types of chronic pancreatitis benefit from being imaged with MRI.

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2014 ADVANCES IN PANCREATITIS

Contemporary review of drug-induced pancreatitis: A different perspective

Whitney Y Hung, Odaliz Abreu Lanfranco

Whitney Y Hung, Department of Pharmacy, Yale New Haven Hospital, New Haven, CT 06510, United States

Odaliz Abreu Lanfranco, Department of Medicine, Yale New Haven Hospital, New Haven, CT 06510, United States

Author contributions: Hung WY and Abreu Lanfranco O solely contributed to this paper.

Correspondence to: Whitney Y Hung, PharmD, BCPS (AQ-ID), Department of Pharmacy, Yale New Haven Hospital, 20 York Street, New Haven, CT 06510, United States. whitney.hung@ynhh.org

Telephone: +1-203-7894211 Fax: +1-203-8675511

Abstract

Although gallstone and alcohol use have been considered the most common causes of acute pancreatitis, hundreds of frequently prescribed medications are associated with this disease state. The true incidence is unknown since there are few population based studies available. The knowledge of drug induced acute pancreatitis is limited by the availability and the quality of the evidence as the majority of data is extrapolated from case reports. Establishing a definitive causal relationship between a drug and acute pancreatitis poses a challenge to clinicians. Several causative agent classification systems are often used to identify the suspected agents. They require regular updates since new drug induced acute pancreatitis cases are reported continuously. In addition, infrequently prescribed medications and herbal medications are often omitted. Furthermore, identification of drug induced acute pancreatitis with new medications often requires accumulation of post market case reports. The unrealistic expectation for a comprehensive list of medications and the multifactorial nature of acute pancreatitis call for a different approach. In this article, we review the potential mechanisms of drug induced acute pancreatitis and provide the perspective of deductive reasoning in order to allow clinicians to identify potential drug induced acute pancreatitis with limited data.

Key words: Drug-induced pancreatitis; Mechanism

Core tip: The knowledge of drug-induced acute pancreatitis (DIAP) is limited by the availability and the quality of the evidence. Potential publication bias may also impact our knowledge of DIAP. Several causative agent classification systems have been proposed, but they require regular updates. In addition, Infrequent prescribed medications and herbal medications are often omitted from those summarized lists. We review the potential mechanisms of DIAP and provide the perspective of deductive reasoning in order to allow clinicians to identify potential DIAP with limited data.

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory condition of the pancreas that may extend to local and distant extra-pancreatic tissues. The annual incidence of AP in the United States is approximately 17 cases per 100000. Acute pancreatitis results in 100000 hospitalizations per year, based on previous reports[1]. An average of 2000 patients per year die from complications related to AP. Although gallstones and alcohol are responsible for more than 90% of all cases in adults, medications have

been recognized as a potential cause of AP[2]. Since the first reported case with chlorthalidone and cortisone in the 1950s, hundreds of commonly prescribed medications from different classes have been reported to induce pancreatic damage. It is expected that the list of drug induced acute pancreatitis (DIAP) will continue to expand with newly approved medications, new cases identified for older agents, and the alternative medicines which have less clinical research support in general. While medications are considered as a common cause of AP, reports of DIAP range from 0.1%-2% of overall cases[2,3].

It is not clear if the true incidence of DIAP has been established due to a lack of mandatory adverse drug report (ADR) system to clinicians, potential publication bias, and the challenge to associate AP with medications. Data from clinical trials of new drugs usually are not informative due to the idiosyncratic character of DIAP. In general, idiosyncratic adverse drug reactions occur with a frequency lower than 1:10000[4]. It is extremely difficult to identify adverse reactions in phase I to phase III clinical investigational trials. Incretin mimetics have been recently introduced in the treatment of diabetes and are widely used in many countries. Incretin mimetics, including exenatide and sitagliptin, were reported to induce AP shortly after those were approved which resulted in warranted an FDA drug safety communication regarding those agents. However, a meta-analysis of randomized clinical trials of most incretin mimetics including sitagliptin did not show any relevant effect on the incidence of pancreatitis. Overall, the incidence was only 0.1% (22 pancreatitis cases found in a pool of 20312 patients). This is an example of the limitation of clinical trials in finding adverse event with low incidence such as DIAP[5].

The knowledge of DIAP is also limited by the availability and the quality of the evidence. It can be difficult to rule out other causes of DIAP, especially in patients who have multiple comorbidities, medications, and underlying risk factors. Since all reports depend on the judgment of the clinicians to exclude other possible causes, reporting more severe ADR has also lead to publication bias. Due to its rarity, most of the evidence comes from case reports of individual drugs and few from case control studies. With a lack of standard ADR reporting format, inadequate data collection in several domains, such as the drug dose, onset of DIAP relative to the use of the medication, and exclusions of other causes, makes it difficult to establish a true causality. In addition, a causal relationship between the agents and DIAP may be difficult to establish due to ethical and practical considerations of re-challenge with the suspected agents. Therefore, the definite relationship between DIAP and medications has only been established in no more than 6% of the agents that have been shown to cause DIAP[6]. Since the identification of DIAP has relied mostly on individual case reports, specific drugs instead of the entire class are usually noted, which makes it even more challenging to identify possible cases in a timely manner.

Potential publication bias may also impact our understanding of DIAP and influence how DIAP is being managed. New drugs or medications with known severe side effects are usually more closely monitored than those that have been in existence for a long time, infrequently prescribed, or considered harmless (*i.e.*, over the counter medications or herbal supplements). Despite the low incidence of drug-induced AP, it is associated with higher morbidity, extended hospital stays, and increased healthcare cost[7]. Approximately 25% of the cases may require intensive care treatment[8]. Developing a systemic approach of identifying potential DIAP is warranted. The aim of this review is to offer a different perspective of approaching DIAP by examining the potential mechanisms of DIAP in order to allow clinicians to identity possible cases with limited data.

ETIOLOGY OF ACUTE PANCREATITIS

Acute pancreatitis is an inflammatory process of the pancreas with varying involvement of other regional tissues or remote organ systems. Gallstone and alcohol use have been considered the most common causes of acute pancreatitis. Gallstone-associated AP is mainly identified by imaging. Previous association with tobacco use is directly linked to alcohol abuse. More evidence associates tobacco use as another toxin that can be directly linked to both acute and recurrent pancreatitis. Other potential mechanical etiologies include periampullary pathologies including intraductal tumor or parasites that are possible in developing countries. In addition to the most common causes, other etiological risk factors for acute pancreatitis are associated with mechanical factors including pancreas divisum, endoscopic retrograde cholangiopancreatography and manometry, as well as trauma or surgical procedures near the pancreas.

Metabolic or systemic process such as hyperlipidemia, infection, and chronic hypercalcemia are well known causes of pancreatitis as well[9]. Infections and toxins, including viral etiologies: mumps, coxsackievirus, hepatitis B, cytomegalovirus, varicella-zoster, herpes simplex virus, human immunodeficiency virus. Bacteria such as *Mycoplasma*, *Legionella*, *Leptospira*, *Salmonella*, *Aspergillus*, *Toxoplasma*, *Cryptosporidium* and *Ascaris* are potential causes of AP. Last, but not least, vascular diseases and pregnancy are also described as causes for pancreatitis.

AP can occur if there is damage to the acinar cells and/or injury to the pancreatic duct that leads to inappropriate accumulation and activation of proenzymes within the pancreas. The activated pancreatic enzymes digest the cell membranes of the pancreas and activate an inflammatory response, which increases the vascular permeability of the pancreas. Hemorrhage, edema, ischemia, and necrosis can result[1,9]. Data from animal studies show that reduced exocytosis and premature fusion of zymogen granules to lysosomes in pancreatic exocrine cells may activate pancreatic proenzymes and lead to cellular autodigestion.

Table 1 Classification system of drug-induced acute pancreatitis according to Badalov *et al*[11]

	Definition	Example
Class I drug	Ia: at least one case report, evidence of a positive re-challenge, and exclusion of other causes of AP	Codeine, cytarabine, dapsone, enalapril, furosemide, isoniazid, mesalamine, metronidazole, pentamidine, pravastatin, procainamide, simvastatin, sulfamethoxazole, sulindac, tetracycline, valproic acid
	Ib: similar to class Ia, except that other causes of AP could not be ruled out	Amiodarone, azathioprine, dexamethasone, ifosfaide, lamivudine, losartan, 6-MP, premarin, TMP-SMZ
Class II drugs	Include at least four case reports with a consistent latency period for at least 75% of the cases	Acetaminophen, Clozapine, DDI, erythromycin, estrogen, l-asparaginase, propofol, tamoxifen
Class III drug	At least two case reports but do not have re-challenge data or a consistent latency period	Alendronate, carbamazepine, ceftriaxone, clarithromycin, cyclosporin, hydrochlorothiazide, interferone/ribavirin, metformin, minocycline, naproxen, paclitaxel, prednisone, prednisolone
Class IV drug	One case report without re-challenge data	Ampicillin, cisplatin, colchicine, cyclophosphamide, diclofenac, doxorubicin, interleukin-2, octreotide, propoxyphene, rifampin, risperidone, sertaline, tacrolimus, vincristine

AP: Acute pancreatitis; 6-MP: 6-mercaptopurine; TMP-SMZ: Trimethoprim and sulfamethoxazole.

CLASSIFICATION OF CAUSATIVE AGENTS

It is difficult to determine if the effects are intrinsic for all members of a drug class despite reports of DIAP incidence within the class. Several classification systems have been proposed. A substantial number of medications are known to cause AP, however, the underlying mechanism is still not well understood. The classification systems rely on summarized lists of medications from previously published reviews to help make the diagnosis of DIAP. Mallory and Kern in 1980s classified drugs that may cause pancreatitis into three groups: definite, probable, or possible association with pancreatitis[5,9]. In order to improve the quality of evidence, different classification systems have also been proposed that categorized DIAP in classes based on the number of reports and re-challenge results[5,10].

Badalov *et al*[11] in 2007 expanded the classification system to five categories: Ia, Ib, II, III, and IV (Table 1). Classifications are based on the published reports from 1955 to 2005. Class Ia includes drugs with at least one case report, evidence of a positive re-challenge, and exclusion of other causes of AP. Class Ib is similar to class Ia, except that other causes of AP could not be ruled out. Criteria for class II drugs include at least four case reports with a consistent latency period for at least 75% of the cases. Class III drugs have at least two case reports but do not have re-challenge data or a consistent latency period. Finally, class IV drugs have one case report without re-challenge data. This classification provides a quick reference of potential causative agents based on the available data at the time of the review. However, regular updates of existing classification are needed since new cases of DIAP are reported continuously. Furthermore, infrequently prescribed medications and alternative medications are often omitted from these summarized lists.

Mechanism of DIAP
The majority of the reported DIAP cases seem to have

an idiosyncratic character. Idiosyncratic reactions to drugs are adverse effects that are not directly related to pharmacodynamic mechanisms of the drugs. These adverse events can occur unpredictably via abnormal interactions between the drugs and the organism, which is usually mediated by immunologic or cytotoxic effects triggered by the drug or its metabolites in a specific organ, in this case, the pancreas[11]. Although the exact mechanism of DIAP is not always known, the pathogenesis should not differ from other causes of AP. It is believed that the pathogenesis of AP differs only in the injury mechanism. It consists of three steps: (1) premature activation of trypsin in acinar cells; (2) intrapancreatic inflammation; and (3) extrapancreatic inflammation[5]. Several mechanisms have been hypothesized including immune-mediated, direct pancreatic toxicity, pancreatic-duct constriction, influence of medication on the bile flow, thrombosis, metabolic effects, and hypersensitivity[12,13]. Mechanisms of DIAP is showed in Figure 1.

Researchers have also used latency to classify the potential mechanisms of DIAP[5]. It is hypothesized that direct immunological effects are usually observed within the first month of drug exposure, whereas toxic effects are noted after a few months of treatment. Potential mechanisms of DIAP include hypersensitivity (onset after four to eight weeks of use), accumulation of a toxic metabolite (onset after several months of use), hypertriglyceridemia (onset after several months of use), and intrinsic toxicity, which is sometimes related to overdose (onset may be almost immediate)[14]. However, there are exceptions that exist. Clinicians should not ignore long lasting medications while DIAP is a concern.

The following reviews summarize five major types of mechanisms of DIAP (Table 2), namely structural, toxins, metabolic, vascular, and other.

Structural
The structural damage such as compression, obstruction, or inflammation of the pancreatic duct may lead to AP. The most common cause for obstruction is choledocholithiasis, or gallstones. Obstruction can also be caused by
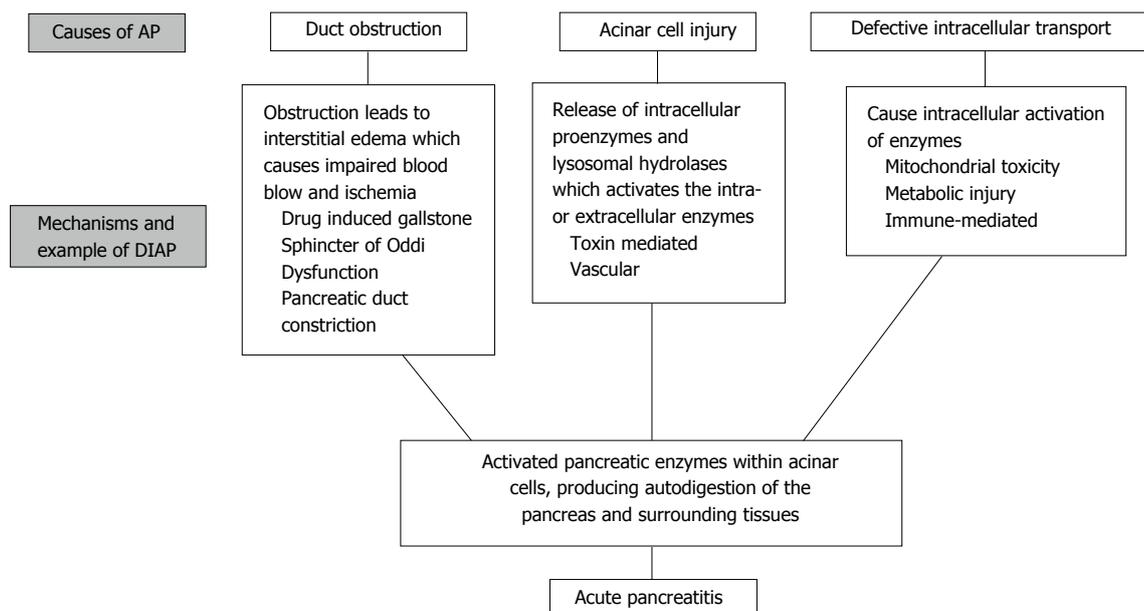
Figure 1 Mechanism of drug-induced pancreatitis. AP: Acute pancreatitis; DIAP: Drug-induced acute pancreatitis.

Table 2 Mechanism of drug induced pancreatitis with drugs associated with acute pancreatitis

Mechanism of DIAP	Drugs with a definite relationship or with class I / II to AP	Probable	Similar structure/class/mechanism with reported cases
Structural	Cholestatic liver injury		Rofecoxib
	Azathioprine		
	Cytarabine		
	Spasm of the sphincter of Oddi	Octreotide	Opium
	Opioids		Marcolides
	Codeine		
	Erythromycin		
	Obstruction		ACE-inhibitors
	Enalapril-angioedema		
	Duct constriction		NSAIDs
	Sulindac		
	Stone		Ceftriaxone
			Dipyridamole
Toxins	Acetaminophen	Metformin	Minocycline
	Didanosine		Tigecycline
	Isoniazid		Doxycycline
	Metronidazole		NRTI
	Valproic acid		HMG-CoA reductase inhibitors
	Mesalamine		
	Pentamidine		
	Asparaginase		
	Sitaliptin		
	Exenatide		
	Tetracycline		
	Pravastatin		
Metabolic	Hypertriglyceridemia	Hydrochlorothiazide	Isotretinoin
	Estrogens	Interferon alfa	Retinoid derivaties
	Corticosteroids	Propofol	Protease inhibitors
	Furosemide	Tamoxifen	Saw palmetto
	β-blocker		Ethacrynic acid
	Clomiphene		Anti-psychotics (aripiprazole, clozapine, olanzapine, quetiapine, risperidone)
	Hypercalcemia		Ⅳ calcium
			Vitamin D
Vascular			Contrast media - iopamidol Procainamide
Immune-mediated	Azathioprine/mercaptopurine		
	sulfasalazine		

AP: Acute pancreatitis; NRTI: Nucleoside reverse transcriptase inhibitor; NSAIDs: Nonsteroidal anti-inflammatory drugs.

duodenal inflammation in Crohn's disease[1].

Medications with risk of gallstones: Ceftriaxone, a third-generation cephalosporin that is excreted from bile duct, has been associated with the development of sludge or stones in the gallbladders for some patients treated with this medication. Secondary pancreatitis has been considered in association with ceftriaxone-induced pseudolithiasis[15]. Unlike ceftriaxone, the kidney pathway is the major means of elimination for most of cephalosporins. It could potentially explain why DIAP has not been reported as class wide induced disease.

Based on an increased amount of cholesterol secreted in bile, causing an increased risk of gallstones which may explain the mechanism of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor-induced AP[10]. Long-term administration of dipyridamole and octreotide can form insoluble substances that precipitate in the gallbladder bile to promote gallstone formation as they are highly excreted from the bile[16,17].

Medications that Cause Sphincter of Oddi Dysfunction: As another example of structural disturbance, the Sphincter of Oddi (SO) is situated at the junction of the bile and pancreatic ducts where they enter the duodenum and serves to regulate the flow of bile and pancreatic juices as well as preventing reflux of duodenal contents into the pancreatobiliary system. SO dysfunction refers to two possible conditions-papillary stenosis (edema or hypertrophy) and dyskinesia (tachyoddia, induced spasm) that lead to partial or complete obstruction of the pancreatic duct resulting in pancreatitis[18]. SO dysfunction is implicated as a cause of various forms of AP including gallstone pancreatitis, pancreatitis secondary to alcohol, scorpion envenomation, and organophosphate poisoning. Medications such as octreotide, opioids, opium, and codeine reportedly induce AP in association with SO dysfunction[19,20]. Erythromycin can cause DIAP due to its prokinetic effect on the smooth muscle of the gastrointestinal track and the gallbladder subsequently increasing the pressure of the SO[21]. Since all macrolide antibiotics have prokinetic effect of different degrees, it is reasonable to consider that AP could potentially be drug related when patients are treated with these agents. As an example, clarithromycin and azithromycin have been reported to be associated with AP[6].

The mechanism of action of the class of drug is also an important factor when evaluating the relatedness of the adverse event to the drug. The probable mechanism of aspirin- or nonsteroidal anti-inflammatory drug (NSAID)- induced pancreatitis is due to inhibition of prostaglandins that otherwise may cause pancreatic duct constriction[12]. Aspirin is shown to increase pancreatic duct permeability in animal models. It increases calcium secretion from the pancreas, which is considered a marker of pancreatic damage. Experimental studies suggested that prostaglandins may have a protective effect on pancreatic cells[22]. Membrane stabilization of pancreatic

cells may be the mechanism behind the cytoprotection conferred by prostaglandins. In NSAID-associated AP, sulindac seems to stand out as the individual drug from the class with the highest number of published cases[23-26].

Toxins

Cumulative dose-dependent effect of toxic metabolites is also hypothesized in drugs showing a consistent long latency (more than 30 d) at the onset of the first episode of DIAP such as valproic acid[5]. Below we discussed a few classic examples of toxin-mediated DIAP.

Nucleoside reverse transcriptase inhibitor: The leading hypothesis of nucleoside reverse transcriptase inhibitor (NRTI)-associated pancreatitis involves mitochondrial toxicity caused by the inhibition of human mitochondrial DNA polymerase-gamma[27]. This inhibition leads to impaired oxidative phosphorylation and failure to synthesize ATP, which is vital for energy-requiring reactions within the cell. Tissues with the highest energy demand appear to be most susceptible. Mitochondrial toxicity is shared among nucleoside analogues and AP attributed to these agents has been described[28]. The degree of mitochondrial impairment and the resultant tissue-specific clinical manifestations vary depending on the NRTI. Among non-nucleoside reverse transcriptase inhibitors, nevirapine is associated with pancreas-related toxicities, whereas efavirenz is not[29,30].

Metronidazole: One speculative mechanism of metronidazole-induced pancreatitis is that under aerobic conditions, it may undergo redox cycling and yield hydrogen peroxide, superoxide, and other free radicals, which can be toxic to pancreatic beta cells and induce pancreatitis[31].

Pentamidine: Pentamidine has a cytotoxic effect on pancreatic β-cells isle and can cause hypoglycemia or hyperglycemia[32]. Same effect can be expected on acinar pancreatic cells.

L-asparaginase: Animal models suggested that L-asparaginase -induced pancreatic injury can involve disruption of the plasma amino acid balance. Disruption of protein synthesis in acinar cells can cause inhibition of exocytosis following the histologic morphologic changes[33].

Tetracycline: Medications in the tetracycline class, including tetracycline, minocycline, and oxytetracycline, are also associated with AP[34-37]. Tetracycline-induced fatty metamorphosis of the liver usually accompanies evidence of pancreatitis, but pancreatitis without evidence of liver disease has also been observed after administration of tetracycline. Steinberg hypothesized that accumulation of an unidentified toxic metabolite may be the cause of tetracycline-induced pancreatitis[38]. Others suggest that high biliary concentration of tetracycline may be associated with tetracycline-induced pancreatitis[39]. Bile concentrations of minocycline after a 200 mg loading dose followed

by one single 100 mg dose were observed to be more than 10 times higher than concurrent serum concentrations (mean serum concentration 0.65 mcg/mL, range 0.07-1.85 mcg/mL)[40]. Tigecycline, the first available member of the glycylcycline group, is a derivate of minocycline and can share similar side effects. Concentrations of tigecycline in bile (median 75.2 mg/L, range 15.9-1150 mg/L) were also found to be several logs greater than concurrent serum concentration (median 0.112 mg/L, range 0.042-0.25 mg/L) after a single 100 mg dose. The mean and median bile-to-serum 24-h area under the concentration-time curve (AUC0-24) ratios were 537 and 368 respectively[41]. It is reasonable to suspect DIAP as a possible complication in tigecycline treated patients. Several cases have been reported previously[42].

Metabolic

Hypertriglyceridemia: It is generally accepted that levels of triglycerides (TG) greater than 1000 mg/dL may increase the risk of precipitating an episode of pancreatitis[43]. The breakdown products of TG are probably responsible for inducing pancreatitis. When lipase in the pancreatic capillary bed acts on the high levels of TG in serum, toxic free fatty acids are generated. The endothelial lining of small pancreatic blood vessels is the first site of injury. Damages of small blood vessels lead to recruitment of inflammatory cells and thrombosis. Hyperlipidemic pancreatitis may be associated with normal serum amylase but with elevated serum lipase levels[44]. With excessive TG, local ischemia and acidemia may occur due to capillary obstruction[45]. This damage exposes TG to pancreatic lipases, which impact degradation of TG[46]. Hydrolysis of TG by pancreatic lipase, excessive formation of free fatty acids with inflammatory changes, capillary injury, and hyperviscosity are postulated to account for the development of hypertriglyceridemia-induced pancreatitis.

Drugs including estrogens, isotretinoin, propofol, retinoid derivatives, HIV protease inhibitors, β-blockers, thiazides, and furosemide are thought to induce AP owing to hypertriglyceridemia. Estrogen is the most well studied drug in this manner. Exogenous estrogens increase serum TG and fatty acids primarily by reducing levels of lipoprotein and hepatic lipases, which subsequently decrease clearance and aggravate insulin resistance[47]. Typically, estrogen-related pancreatitis occurs within the first months following estrogen initiation. Obese patients with underlying glucose intolerance or fasting hypertriglyceridemia are at greater risk[44]. However, reports have also shown that estrogen-associated DIAP can happen without elevated serum lipid concentrations[48,49]. It is thought that arteriolar thrombosis may be another potential mechanism of action[48,50]. Tamoxifen and clomiphene are synthetic estrogen analogues with mixed agonist-antagonist actions. Cases of tamoxifen- or clomiphene-associated AP have been reported with mechanisms similar to that of estrogen.

Dibenzodiazepine-derived atypical antipsychotics (*i.e.*, clozapine, olanzapine, and quetiapine) may also be a potential cause of DIAP. Both risperidone and ziprasidone are non-dibenzodiazepine atypical antipsychotics and appear to have minimal effect on serum lipids[51]. This is another example where clinicians can apply the general knowledge of each medication when evaluating the likelihood of DIAP for the newer medications.

The previous section discussed that tetracyclines-associated DIAP due to its toxic metabolite and high biliary concentrations. Elmore and Rogge[36] also proposed a tetracycline-induced hypertriglyceridemia mechanism with subsequent pancreatitis. Tetracycline inhibits protein synthesis by binding to the 30S ribosomal subunit in the messenger ribonucleic acid (mRNA) translation complex. Blockage of protein synthesis could result in accumulation of defective proteins within hepatocytes. This inhibits the release of TG from the liver, which may lead to pancreatitis.

Hypercalcemia: Calcium is identified as the most important intracellular element in acinar cell stimulus-secretion coupling[52]. Disruption in the secretory process could be the mechanism by which hypercalcemia induces pancreatitis. Based on experimental studies, increase in extracellular calcium leads to a functional secretory block with dose-dependent characteristics[53]. Acinar cell stimulation induces spikes in cytosolic calcium concentration by repetitively releasing calcium from intracellular stores, which activates the normal secretory process of digestive enzymes from intracellular zymogen stores. Excessive extracellular calcium concentration leads to sustained increases in cytosolic calcium. It results in vacuole formation and trypsinogen activation and eventually leads to edematous or necrotizing pancreatitis[54]. Research indicates that hypercalcemia is associated with an increase in serum enzymes[44]. Intravenous calcium administration has been associated with pancreatitis in at least two published reports. Additionally, pancreatitis has been correlated to cases of vitamin D poisoning and to patients receiving total parenteral nutrition[55]. It is suspected that all drugs which can cause hypercalcemia carry risk of inducing AP.

Thiazides, a class with hypertriglyceridemia potential, could also induce hypercalcemia and hypophosphatemia. Thiazide-induced reductions in blood pressure may lead to pancreatic ischemia. They may act directly on the pancreas or indirectly by altering calcium metabolism. Therefore, there are multiple mechanisms exhibited by thiazides that could potentially lead to AP.

Vascular

Ischemia is an uncommon cause of AP. Pancreatic infarcts may occur in patients with underlying atherosclerotic vascular disease, but they are unusual because the pancreas is richly perfused from several different arterial sources. Cholesterol emboli may cause pancreatitis, cholecystitis, or bowel ulceration or infarction, and should be suspected when AP occurs after vascular interventions such as cardiac catheterization. Patients may have associated evidence

of renal, gut, or peripheral cholesterol emboli. Ischemic pancreatic and hepatic injury may be associated with malignant hypertension, low flow states due to severe heart failure, or administration of potent vasoconstrictors. Vasculitis may cause pancreatitis associated with systemic autoimmune diseases. Acute pancreatitis secondary to drug-induced lupus syndrome has also been described[56].

Contrast-induced pancreatitis may be related to decreased oxygenation and impaired circulation of the pancreas. Iopamidol has a viscosity of 9.4 cP at 37 degrees centigrade versus human plasma of 1.72 cP at hematocrit of 43%. A similar pathophysiologic process has been proposed in contrast-induced kidney injury. Cholesterol crystal embolization may be another mechanism that results in occlusion of small arteries[57].

Immune-mediated reaction

Direct immunological effects are usually observed within the first month of drug exposure, whereas toxic effects are noted after a few months of treatment[45]. Researchers have also considered AP an immune-mediated reaction if relapse occurs rapidly after re-challenge as seen with sulfonamides and aminosalicylates (*e.g.*, sulfasalazine and mesalazine)[58-60]. The latency between initiation of the drug and the onset of DIAP is usually one week to a month, but reexposure can lead to a new episode in one to three days[5]. Cases of azathioprine or the thiopurine bases mercaptopurine-induced pancreatitis are well documented. Studies have shown patients with decreased levels of thiopurine metabolizing enzyme inosine triphosphate pyrophosphatase may be at an increased risk of developing thiopurine-induced AP. However, 6-tioguanine-induced pancreatitis is less common than conventional thiopurine. Only 1% of inflammatory bowel disease (IBD) patients previously intolerant to the conventional thiopurines are reported to have 6-tioguanine-induced AP after treatment[61]. A strong correlation with immune disorders, mainly Crohn's disease and HIV infections, implies an immune-mediated reaction as a chief causative factor of the disease.

Angiotensin-converting-enzyme inhibitors: Captopril, enalapril, lisinopril, perindopril, benazepril, and quinapril have all been associated with AP[25,52]. Pancreatic duct obstruction by local angioedema may be the mechanism by which angiotensin-converting-enzyme-inhibitors cause pancreatitis. Others propose a direct toxic effect on pancreatic cells. Since captopril is structurally dissimilar to enalapril and lisinopril, an allergic reaction seems less likely. Angiotensin receptor blockers may share a similar mechanism for pancreatitis, at this point definitive cases are not described in literature[10].

Alternative medicines including herbal medication

There are very limited data of DIAP associated with herbal or over the counter medications when compared to prescription medications. Although a mechanism for saw palmetto-induced AP has not been thoroughly estab-

lished, cases of saw palmetto-induced cholestatic hepatitis associated with AP have been reported. Currently, there are only two reported cases of saw palmetto induced AP. Another theory suggests that it occurs through its estrogenic effects by stimulating estrogen receptors and then induces a hypercoagulable state that leads to pancreatic necrosis[2]. This information should prompt clinicians to consider saw palmetto a potential cause of AP.

A WORK UP FOR ACUTE PANCREATITIS

Since no specific test for establishing the diagnosis of DIAP is available, the diagnosis is usually based on excluding all other common causes. Pancreatitis is suspected when a patient presents with clinical features including acute onset of persistent and severe epigastric abdominal pain, and is then confirmed by laboratory and imaging studies that exclude other serious intra-abdominal conditions. Most patients will have elevations in serum levels of amylase or lipase within a few hours of the onset of symptoms. Lipase tends to remain elevated longer than amylase. Amylase and lipase levels above three times the upper limit of normal are mostly associated with pancreatitis. Once these levels are elevated, serial measurements are of no clinical significance for prognosis or outcomes. They should not be obtained routinely after the initial measurements are obtained.

Imaging studies are used to establish the diagnosis, but also to determine etiology and prognosis. Both abdominal ultrasound and abdominal computed tomography (CT) can be used interchangeably; however, the latter is preferred as it can provide alternative diagnoses.

As part of the investigation for potential causes of AP, a history of alcohol/tobacco use, previous biliary colic, medication history, family history, and recent trauma should be elicited. Gallstone-associated pancreatitis should be suspected if stones are seen on imaging studies or if liver chemistries are abnormal and then improve over a few days. A three-fold elevation of ALT has a high predictive value for gallstone-associated AP. Hypertriglyceridemia, especially with levels above 1000 mg/dL, and hypercalcemia can be evaluated based on laboratory data. Infections, including viral etiologies, are potential causes of AP as well.

A suspected drug etiology should be considered after the exclusion of more common causes of illness. As mentioned above, it is challenging to establish the causality between medications and associated AP. The use of classification systems may be useful as the first screening tool. Since the mean interval between initial drug administration and start of the symptoms is approximately 5 wk, with a range of 2 to 36 wk[46], clinicians can target those medications when reviewing the medication profiles. If the mediations are not listed on these summarized lists, clinicians should identify if similar structured medications have been associated with DIAP and evaluate the possibility of sharing a similar mechanism of inducing pancreatitis. Once the target agent is identified, the offending agent
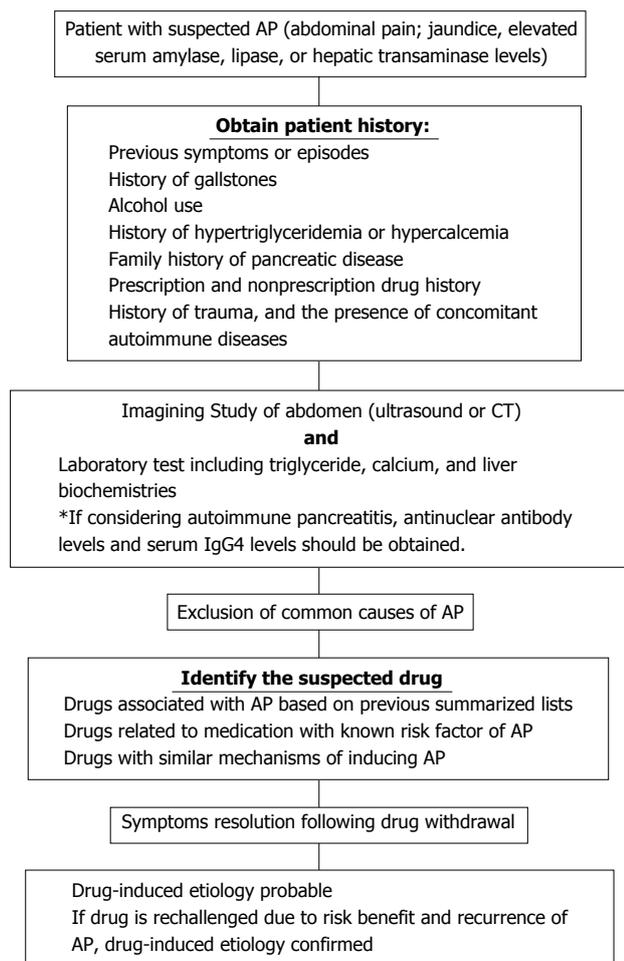
Figure 2 Algorithm of Identifying A potential case of drug induced acute pancreatitis. AP: Acute pancreatitis.

should be discontinued, preferably one at the time to avoid confounders. Most reactions are reversible and resolve on their own within 3-7 d after the offending agent has been discontinued. Due to the nature of the disease state and ethical consideration, re-challenge of the suspected drug is usually not possible. Often times, the medication can be just a possible/probable cause of AP. If re-challenge of the suspect drug is considered necessary, the patient's written informed consent should be obtained. An algorithm of identifying a potential case of drug induced AP is presented in Figure 2.

DISCUSSION

Hundreds of medications have been suggested to be the potential cause of AP, although the true incidence of DIAP is unknown. Evidence associating drugs with AP is largely based on individual cases. It is unrealistic to expect a comprehensive list that includes all agents associated with AP due to continuously reported new cases. Although relapse of pancreatitis after controlled re-challenge confirms a causal relationship, such proof is uncommon. Furthermore, re-challenge is only ethical when the same treatment is absolutely necessary for the

patient. It leads to only few causal relationships for the reported agents.

Few causative agent classifications have been proposed. These classifications have helped clinicians understand the quality of evidence behind each potential causative agent. However, with the exception of a few agents with a definite relationship confirmed by re-challenge, it depends on each individual report to exclude all other possible causes, especially drug effects that may be difficult to separate from the underlying conditions. Clinically, certain subpopulations such as children, women, the elderly, patients with IBD and patients with HIV appear to be at a higher risk[2]. Mesalazine, azathioprine, and corticosteroids, for instance, are used in the treatment of IBD which itself increases the risk of AP. Anti-retroviral agents is another example as HIV is an independent risk factor of AP. A study that compared patients with and without HIV infection found a drug-related etiology in 41% and 5% of the patients with AP respectively[62]. The use of NRTIs such as didanosine, stavudine, and lamivudine and co-administration of other medications such as pentamidine, cotrimoxazole, antimycobacterial therapy, or cytotoxic chemotherapy for at least 6 mo were found to be a significant risk factor for at least a three-fold increase in serum pancreatic enzymes ($P < 0.05$). Certain medications, such as proton pump inhibitors and histamine2-receptor antagonists as well as NSAIDs, may be initiated in response to early symptoms of unrecognized pancreatitis. This may have led to erroneously attributing the pancreatitis to these medications[63,64]. Repeated cases of DIAP are more likely to be published or even diagnosed than those without prior reports. Due to underreporting incidence rates from spontaneous reports and potential publication bias with only reporting severe cases, it has further complicated the assessment of the causal relationship between drugs and AP based on current proposed classification.

Efforts have been devoted to improve drug safety surveillance strategies. Vilar *et al*[65] have shown promising results of detecting adverse drug events related to pancreatitis by developing molecular fingerprint-based models. The models were based on the premise that similar molecules can have comparable biological properties. For example, tigecycline is structurally related to minocycline and shares similar pharmacokinetic properties and side effects with tetracyclines. Not surprisingly, cases of tigecycline-induced AP were reported soon after its introduction to the market[42]. Nevertheless, DIAP is generally not considered as a drug class effect, so specific drugs are usually noted instead of the entire class[14]. It is suggested that clinicians take the potential mechanism of DIAP into account. For example, ceftriaxone has different pharmacokinetic properties than other cephalosporins and may lead to secondary pancreatitis caused by only ceftriaxone induced pseudolithiasis.

Limited data exist regarding the mechanisms of DIAP. The pathogenesis is not completely understood. Nevertheless, DIAP should not have unique features that

distinguish it from AP due to other causes. Drugs may lead to pancreatitis by inducing known risk factors of AP such as structural (*e.g.*, cholestatic liver injury, spasm of the SO, duct obstruction/constriction, and stones), metabolic (*e.g.*, hypertriglyceridemia and hypercalcemia), and vascular effects. Some drugs or drug metabolites may theoretically have a direct toxic effect on the pancreas. Other than known mechanisms of toxicity such as mitochondrial toxicity and protein synthesis inhibition, the high level of gastrointestinal drug concentration may be needed to cause cytotoxic damage. Drugs with a definite causal relationship to AP including isoniazid, metronidazole, valproic acid, mesalamine, and tetracycline share similar pharmacokinetic properties by extensive hepatic metabolism. If other potential causes of DIAP have been ruled out, drugs that are highly concentrated in the gastrointestinal tract could be potential suspects of DIAP. For other drugs, an immunoallergic idiosyncratic reaction is more likely. Re-challenge with these drugs usually leads to prompt recurrence of symptoms in a dose-independent manner. In an animal study, the results suggest that DIAP is multifactorial and may explain why the incidence of DIAP is low[18].

Establishing a definitive causal relationship between a drug and AP poses a challenge to clinicians. Depending on the agents, the time from the initiation of therapy to the onset of pancreatitis symptoms varies. Pancreatitis can occur within a short time after administration of the first dose to years after therapy begins for most of the drugs. The unrealistic expectation of the comprehensive list and the multifactorial natures of the causes of AP call for a different approach. This article reviews the potential mechanisms of DIAP and provides the perspective of deductive reasoning in order to identify potential DIAP.

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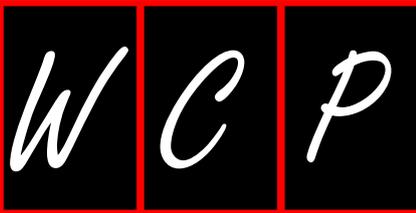
Acute pancreatitis in children and adolescents

Mitsuyoshi Suzuki, Jin Kan Sai, Toshiaki Shimizu

Mitsuyoshi Suzuki, Toshiaki Shimizu, Departments of Pediatrics, Juntendo University, Tokyo 113 8421, Japan
Jin Kan Sai, Departments of Gastroenterology, Juntendo University, Tokyo 113 8421, Japan
Author contributions: Suzuki M performed experiments and participated in writing and figure creation; Sai JK and Shimizu T conceived the idea and participated in writing.
Correspondence to: Mitsuyoshi Suzuki, MD, PhD, Department of Pediatrics, Juntendo University, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113 8421, Japan. msuzuki@juntendo.ac.jp
Telephone: +81-3-38133111-3640 Fax: +81-3-58001580
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Abstract

In this Topic Highlight, the causes, diagnosis, and treatment of acute pancreatitis in children are discussed. Acute pancreatitis should be considered during the differential diagnosis of abdominal pain in children and requires prompt treatment because it may become life-threatening. The etiology, clinical manifestations, and course of acute pancreatitis in children are often different than in adults. Therefore, the specific features of acute pancreatitis in children must be considered. The etiology of acute pancreatitis in children is often drugs, infections, trauma, or anatomic abnormalities. Diagnosis is based on clinical symptoms (such as abdominal pain and vomiting), serum pancreatic enzyme levels, and imaging studies. Several scoring systems have been proposed for the assessment of severity, which is useful for selecting treatments and predicting prognosis. The basic pathogenesis of acute pancreatitis does not greatly differ between adults and children, and the treatments for adults and children are similar. In large part, our understanding of the pathology, optimal treatment, assessment of severity, and outcome of acute pancreatitis in children is taken from the adult literature. However, we often find that the common management of adult pancreatitis is difficult to apply to children. With advances in diagnostic techniques and treatment methods, severe acute pancreatitis in children is becoming better understood and more controllable.

Key words: Acute pancreatitis; Children; Pathophysiology; Etiology; Diagnosis; Treatment

Core tip: The etiology, manifestations, and course of acute pancreatitis in children are often different than in adults, and these differences should be highlighted. The etiology of acute pancreatitis in children is drugs, infections, trauma, or anatomic abnormalities. The diagnosis of acute pancreatitis is based on clinical symptoms, serum pancreatic enzyme levels, and imaging studies. Treatments in adults and children are similar. With advances in diagnostic techniques and treatments, severe acute pancreatitis in children is becoming better understood and more controllable.

INTRODUCTION

Acute pancreatitis is not necessarily a rare disease, even in children and adolescents (hereinafter referred to as "children"), and may be life-threatening if it is severe[1,2]. Therefore, acute pancreatitis should always be considered during the differential diagnosis of abdominal pain in children, and appropriate treatment should be started promptly when necessary. However, many treatment regimens are based on consensus conferences and evidence in adults, so a search for the cause and appropriate treatment in children is often difficult[3,4]. This paper discusses the causes, diagnosis, and treatment of acute pancreatitis in children, including a review based on our own experiences.

Table 1 Etiology of childhood acute pancreatitis

Congenital anomalies, periampullary obstruction
  Choledochal cyst, abnormal union of the pancreaticobiliary junction, gallstone, cholecystitis, pancreatic divisum, tumor, ascaris aberrant
Infectious
  Mumps, measles, coxsackie, echo, lota, influenza, epstein-barr virus, Mycoplasma, salmonella, gram-negative bacteria
Drugs
  L-asparaginase, steroid, valproic acid, azathioprine, Mercaptopurine, mesalazine, Cytarabine, Salicylic acid, indomethacin, tetracycline, chlorothiazide, isoniazid, anticoagulant drug, borate, alcohol
Trauma
  Blunt injury, child abuse, ERCR, After surgery
Systemic disease
  Reye syndrom, systemic lupus erythematosus, polyarteritis nodosa, Juvenile rheumatoid arthritis, sepsis, multiple organ failure, Organ transplantation, hemolytic-uremic syndrome, henoch-schoenlein purpura, kawasaki disease, inflammatory bowel disease, chronic intestinal pseudo-obstruction, gastric ulcer, anorexia nervosa, food allergy, cystic fibrosis
Metabolic
  Hyperlipoproteinemia (I, IV, V), hypercalcemia, diabetes, α1 antitrypsin deficiency
Nutrition
  Malnutrition, high-calorie infusion, vitamin A and D deficiency
Others
  Familial, idiopathic

ERCP: Endoscopic retrograde cholangiopancreatography.

Table 2 Cause of acute pancreatitis in children and adolescents

Ref.	Location	Cases	Etiology (%) Systemic	Biliary[1]	Anatomic[2]	Trauma	Familial	Metabolic[3]	Drugs	Others[4]	Idiopathic
Lopez[50]	United States	274	48	10	NA	19	NA	0.7	5	0.4	17
DeBanto *et al*[1]	United States	301	3.5	10.5	1.5	13.5	5.5	4	11	16.5	34
Werlin *et al*[8]	United States	180	14	12	7.5	14	3	5.5	12	24	8
Nydegger *et al*[4]	Australia	279	22.2	5.4	NA	36.3	NA	5.8	3.2	2.2	25.1
Suzuki *et al*[19]	Japan	135	8.9	30.4	25.9	9.6	NA	NA	11.1	3.7	10.4
Lantz *et al*[2]	United States	211	3.3	11.8	5.2	7.6	0.9	6.2	19.9	13.8	31.3

All studies contained more than 100 cases. NA: Not available. [1]Gallstone, biliary sludge, choledochal cyst; [2]Abnormal union of the pancreaticobiliary junction, pancreatic divisum; [3]Diabetic acidosis, hyperlipidemia, organic acidemias, hypercalcemia; [4]Associated viral infection, postendoscopic retrograde cholangiopancreatography, alcohol, autoimmune, cystic fibrosis, post-surgery.

ETIOLOGY

Alcohol and gallstones are the etiology of acute pancreatitis in many adults, and although some differences exist based on sex and ethnicity, these two etiologies account for more than 60% of cases of acute pancreatitis in adults[5,6]. However, the etiology in children is often drugs, infections, trauma, and anatomic anomalies such as choledochal cysts and abnormal union of the pancreatobiliary junction (Table 1)[1,4,7,8]. Table 2 shows the incidence of acute pancreatitis by etiology. There is a considerable difference in the etiology of acute pancreatitis in Western and Asian children[9].

Drugs

Among drugs used in childhood and adolescence, L-asparaginase (ASNase), steroids, and valproic acid often cause pancreatitis as an adverse reaction. In particular, ASNase, a key drug used in treatment of childhood leukemia, is associated with a higher incidence of pancreatitis as compared to other drugs, ranging from 2%-16% when mild cases are included[10-12]. A characteristic of pancreatitis associated with ASNase, in addition to clinical symptoms of abdominal pain and tenderness, is the early absence of elevated serum amylase levels in about half of patients[13,14]. This phenomenon is attributed to inhibition of protein synthesis by ASNase[14]. Therefore, when acute pancreatitis is suspected based on clinical findings, even in the absence of serum amylase elevation, acute pancreatitis must always be considered in the differential diagnosis, and it is important not to miss the opportunity for early treatment. Azathioprine and mesalazine can also cause pancreatic toxicity, so if serum pancreatic enzyme levels increase during the treatment of inflammatory bowel disease, drug-related pancreatitis must also be considered[15].

Infectious disease

Mumps is often encountered in daily clinical practice, but few patients develop pancreatitis that requires additional treatment. Pancreatitis as a complication is reported in 0.3%-15% of patients when mild cases are included[16]. Abdominal symptoms such as pain and tenderness may occur before the clinical onset of mumps (4-8 d after viral infection) and often spontaneously resolve in about 1 wk. In addition, pancreatitis may occur without parotid
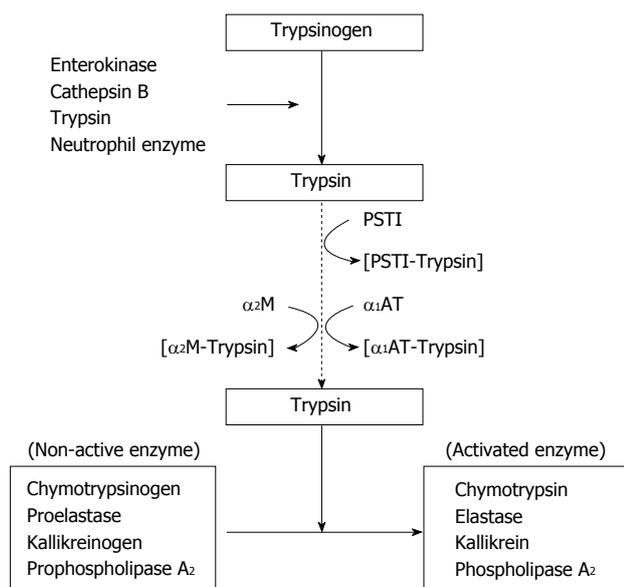
Figure 1 Suppression mechanisms for pancreatic enzyme activation. PSTI: Pancreatic secretory trypsin inhibitor; α_2M: α_2-macroglobulin; α_1AT: α_1-antitrypsin.

gland swelling in a few patients. When pancreatitis of unknown etiology occurs, testing for the mumps virus is recommended. Two deaths have been reported to date, so although rare, possible serious infection must be kept in mind[17].

Pancreatitis associated with mycoplasma infection is broadly classified into two types: early onset type during early infection (days 1-3) and late-onset type after respiratory tract symptoms have occurred (days 7-14). The mechanism in the former is thought to be direct invasion of mycoplasma into the pancreas, and in the latter, pancreatic injury caused by autoantibodies to acinar cells[18]. The prognosis in pancreatitis due to mycoplasma is generally good.

Congenital anomalies

Among anomalies of the pancreatobiliary system, choledochal cyst is the most common cause of acute pancreatitis[1,2,4,19]. In fact, many choledochal cysts are discovered because of symptoms of acute pancreatitis. In children with acute pancreatitis in whom the etiology is unclear, ultrasonography, endoscopic retrograde cholangiopancreatography (ERCP), or magnetic resonance cholangiopancreatography (MRCP) should be performed[20,21]. Most choledochal cysts, with the exception of Todani classification type II (bile duct diverticulum) and type III (choledochocele), are associated with abnormal union[22]. The sphincter of Oddi is usually most thickened in the duodenal muscularis mucosa; however, in abnormal union, because this sphincter surrounds a common channel after union of the main pancreatic duct and common bile duct, there is communication between the ducts during sphincter contraction[23]. Therefore, reflux of bile into the pancreatic duct, a protein plug in the common channel, or gallstone impaction is probably involved in the onset of pancreatitis.

PANCREATITIS CAUSED BY GENETIC MUTATIONS

Hereditary pancreatitis is due to autosomal dominant inheritance with about 80% penetrance. A relationship between a mutation in the cationic trypsinogen gene (protease serine 1, *PRSS1*) and hereditary pancreatitis was identified in 1996[24]. In 2000, a mutation in the serine protease inhibitor gene (*Kazal* type 1: *SPINK1*) was reported to be related to chronic idiopathic pancreatitis of unknown cause[25]. Patients with hereditary pancreatitis due to a *PRSS1* gene mutation or relapsing pancreatitis due to a *SPINK1* gene mutation can develop pancreatic exocrine insufficiency and diabetes in the future, and they are a high-risk group for pancreatic cancer[26-28]. The cause of these complications like cancer, as in chronic pancreatitis due to other etiologies, involves hyperplasia and metaplasia of the pancreatic duct epithelium due to recurrent or chronic inflammation. *K-ras* gene mutations also play a role[29]. Diabetes or pancreatic cancer developing in childhood cases has not been reported.

Recently, variants in *CPA1*, which encodes carboxypeptidase A1, were implicated in early onset pancreatitis in children up to 10 years old. The mechanism by which *CPA1* variants confer increased pancreatitis risk may involve misfolding-induced endoplasmic reticulum stress rather than elevated trypsin activity[30].

Other causes

In malignant lymphoma, lymphoma invasion near the head of the pancreas may compress the pancreatic duct and lead to acute pancreatitis[31]. In addition, in solid pseudopapillary neoplasms, intratumoral hemorrhage due to trauma can cause transient tumor enlargement, leading to pancreatic duct obstruction and acute pancreatitis[32].

PATHOPHYSIOLOGY

To understand the pathophysiology of acute pancreatitis, knowledge about the inhibitory mechanisms of activation of pancreatic enzymes under physiological conditions is necessary. In normal pancreatic acinar cells, lysosomes containing cathepsin B, which are involved in intracellular and extracellular digestion, and zymogen granules containing digestive proenzymes, such as trypsinogen, are released; and these inactive proenzymes remain inactivated[33,34]. In addition, even if trypsin is aberrantly activated in the pancreas for some reason, its activity is blocked by pancreatic secretory trypsin inhibitor (PSTI). Moreover, if trypsin leaks into the blood, the endogenous trypsin inhibitors α_1-antitrypsin (α_1AT) and α_2-macroglobulin (α_2M) bind to trypsin and suppress its activity (Figure 1)[35]. Anatomically, the sphincter of Oddi located in the duodenal ampulla of Vater prevents reflux of duodenal fluid into the pancreatic duct. Pancreatic duct pressure is also usually higher than bile duct pressure, so there is no bile reflux into the pancreatic duct[23].

Excessive stimulation of pancreatic exocrine secre-
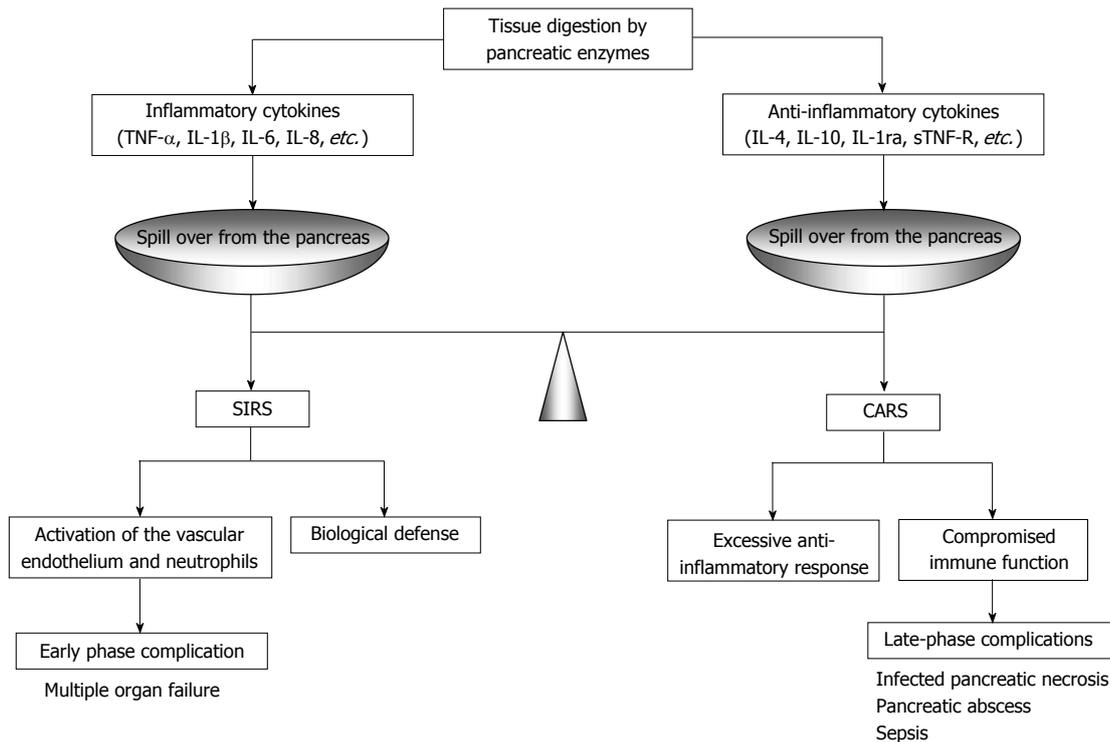
Figure 2 Compensatory anti-inflammatory response syndrome and systemic inflammatory response syndrome during acute pancreatitis. TNF: Tumor necrosis factor; IL: Interleukin; sTNF-R: Soluble tumor necrosis factor receptor; CARS: Compensatory anti-inflammatory response syndrome; SIRS: Systemic inflammatory response syndrome.

tions can cause reflux of pancreatic juices and entero-kinase, pancreatic duct obstruction, and inflammation. These conditions can disrupt the above-mentioned defense mechanisms, activate trypsin beyond the ability for trypsin inactivation, and increase attacking factors, thus leading to acute pancreatitis[36]. Enterokinase is the most efficient activator, but trypsin itself, lysosomal enzymes (cathepsin B) in pancreatic acinar cells, and neutrophilic enzymes are also activators[34,36]. In experimental models of early acute pancreatitis, blockage of secretion has been suggested as the initiating event, leading to the accumulation of zymogen granules within acinar cells. This event is followed by a co-localization of digestive enzymes and lysosomal enzymes within vacuoles and, finally, an activation of enzymes that cause acute intracellular injury[37]. The activation of zymogen protease in pancreatic acinar cells is thought to play an important role in the development of acute pancreatitis[36,38].

Mild pancreatitis mainly involves the pancreas and local surrounding lesions. It is generally reversible, and about 6 mo after clinical remission, the pancreas recovers its normal morphology and function. In severe pancreatitis, vasoactive substances such as histamine and bradykinin are produced in large amounts with trypsin activation. As this vasoactive process increases, third spacing of fluids and shock due to hypovolemia may occur. In addition, leakage of activated enzymes from the pancreas causes secondary cytokine production. These cytokines trigger the systemic inflammatory response syndrome (SIRS)[39,40]. SIRS results in hyperactivation of macrophages and neutrophils throughout the body and the release of tissue

injury mediators; multiorgan failure, including shock, circulatory failure, and acute respiratory distress syndrome (ARDS), may occur[41-43].

Meanwhile, as a biological defense response, anti-inflammatory cytokines and cytokine antagonists are induced to prevent prolongation of SIRS. This predominance of cytokine antagonists is called compensatory anti-inflammatory response syndrome (CARS)[44]. Because CARS inhibits new cytokine production, susceptibility to infection is increased, and infection of vital organs can occur. As a result of infection, endotoxins in the blood stimulate neutrophil aggregation in distal organs, tissue injury mediators are released, and distal organ failure occurs (Figure 2).

CLINICAL DIAGNOSIS AND ASSESSMENT OF SEVERITY

The diagnosis of acute pancreatitis is in principle based on clinical findings, biochemical tests, and imaging studies. Both a differential diagnosis and assessment of severity are necessary. The etiology of acute pancreatitis in children often differs from that in adults, and differences in the clinical manifestations and course may occur. Therefore, the diagnosis should be made keeping in mind specific features of the disease in children and after obtaining a past medical and family medical history (Figure 3).

Clinical manifestations

More than 90% of adults with acute pancreatitis report
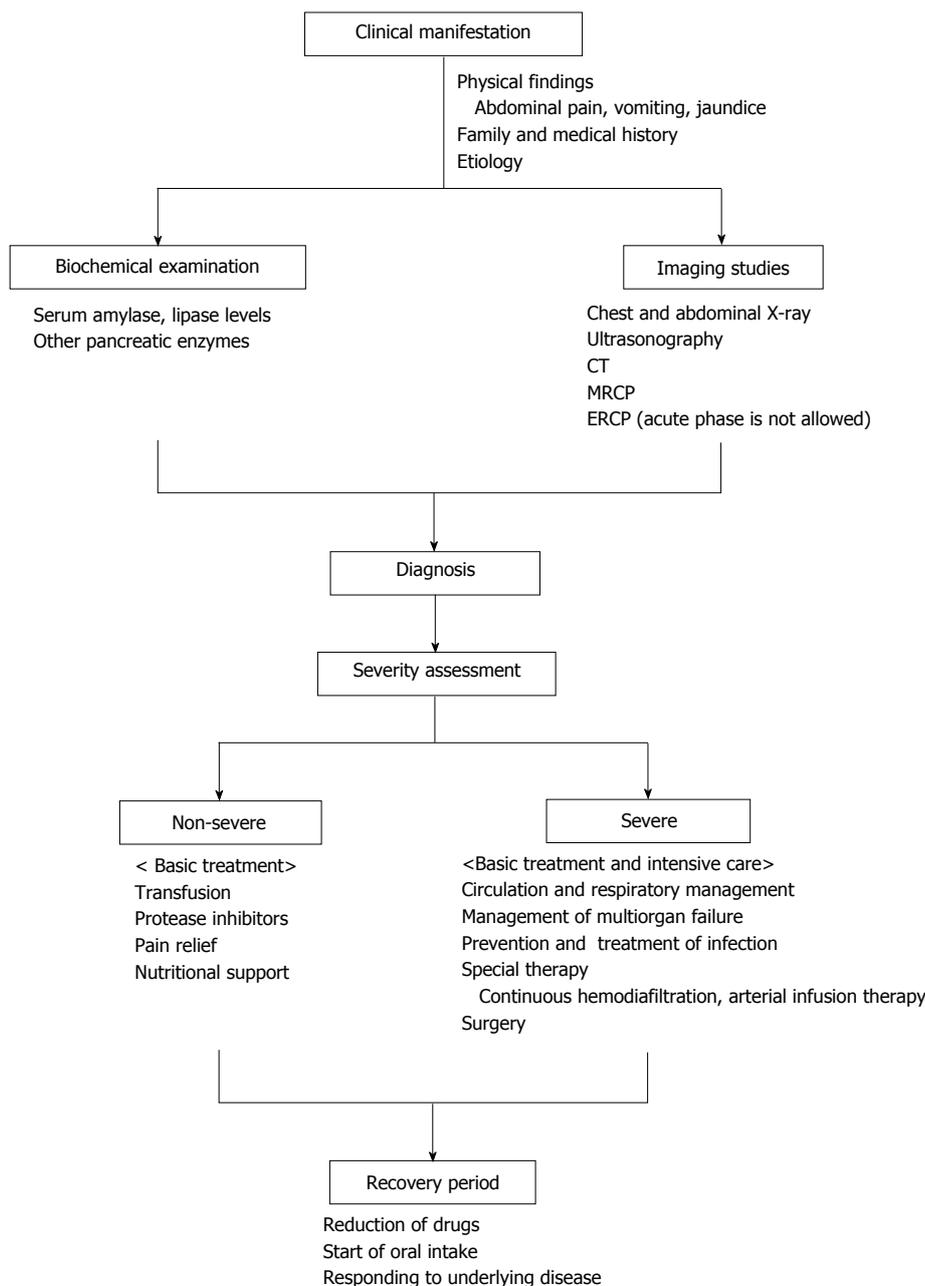
Figure 3 Clinical diagnosis of acute pancreatitis. CT: Computed tomography; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

abdominal pain[45,46]. Abdominal pain is also an important early symptom in children. Weizman *et al*[47] reported that all 61 of their pediatric patients with acute pancreatitis initially had abdominal pain. Ziegler *et al*[48] also reported abdominal pain in 40 of 49 patients (82%). Table 3 shows the initial symptoms by age in our series of 135 children with acute pancreatitis[19]. In older children, the frequency of abdominal pain as a first symptom was similar to that in adults, whereas in younger children, vomiting was an important clinical symptom[49]. However, very young children and those with mild pancreatitis sometimes have non-specific abdominal pain. The location, characteristics, and triggers of abdominal pain, as well as physical examination of the abdomen, are important clues in the diagnosis of acute pancreatitis.

Other symptoms may include jaundice, fever, diarrhea, back pain, irritability, and lethargy. Jaundice and clay-colored stools suggest an abnormality of the biliary system such as a choledochal cyst, and there may be a palpable abdominal mass[8]. Infants and toddlers cannot verbalize abdominal pain, but vomiting, irritability, and lethargy are common[48]. In severe acute pancreatitis, children may initially present with shock, followed by symptoms of multiorgan failure, including dyspnea, oliguria, hemorrhage, and mental status changes[1].

Laboratory investigations
The prompt measurement of serum amylase is useful for

	Age, yr			
	1-5 (*n* = 53)	6-10 (*n* = 47)	11-17 (*n* = 35)	Total (*n* = 135)
Abdominal pain	46 (86.8)	39 (83.0)	32 (91.4)	116 (85.9)
Fever	21 (39.6)	21 (44.7)	10 (28.6)	52 (38.5)
Vomiting	29 (54.7)	16 (34)	6 (17.1)	51 (37.8)
Jaundice	9 (17)	2 (4.3)	0	11 (8.1)
Back pain	0	1 (2.1)	5 (14.3)	6 (4.4)
Pale stool	3 (5.7)	1 (2.1)	0	4 (3)
Diarrhea	0	1 (2.1)	2 (5.7)	3 (2.2)
Loss of consciousness	1 (1.9)	1 (2.1)	1 (2.0)	3 (2.2)
Others	5 (9.5)	2 (4.2)	2 (5.8)	9 (6.6)

Table 3 First symptoms and chief complaints by age *n* (%)

a diagnosis of acute pancreatitis[50]. However, elevated levels are also seen in gastrointestinal diseases such as pancreatobiliary tract obstruction and perforative peritonitis, as well as in salivary gland disease and renal failure. Therefore, low disease specificity is a problem. Serum lipase has a sensitivity of 86.5%-100% and specificity of 84.7%-99.0% for diagnosing acute pancreatitis[51]. Thus, its sensitivity is higher compared to serum amylase. In severe pancreatitis, serum lipase levels 7 times higher than normal have been reported within 24 h after onset of pancreatitis[52]. The degree of elevation and serial changes, however, generally do not correlate with disease severity[53]. In acute pancreatitis due to ASNase or valproic acid, which is fairly common in children, serum amylase may not be elevated[13]. Therefore, other serum pancreatic enzymes should also be measured.

Imaging

When acute pancreatitis is suspected, plain chest and abdominal X-rays are essential. A plain chest X-ray may show a pleural effusion, ARDS, or pneumonia. Although these findings are not specific for acute pancreatitis, they are important for the assessment of disease severity. A plain abdominal X-ray may show an ileus, colon cut-off sign, sentinel loop sign, calcified gallstones, pancreatic stones, or retroperitoneal gas. This information is important in assessing the clinical course of acute pancreatitis and is necessary for a differential diagnosis to rule out other diseases such as gastrointestinal perforation[54,55].

Ultrasonography is a convenient and non-invasive test. It is the test of first choice for screening to diagnose acute pancreatitis in children and for following the clinical course. The ultrasound diagnosis of acute pancreatitis is based on pancreatic morphology, appearance of the pancreatic parenchyma and pancreatic duct, and extrapancreatic findings[56,57].

CT scanning together with ultrasonography is essential for diagnosing acute pancreatitis. CT is useful to evaluate any extrapancreatic lesions, monitor the clinical course, and assess severity. In particular, CT is superior for early assessment of acute pancreatitis when ultrasound findings are nonspecific because of abdominal gas[56,58].

Pancreatitis in children is often caused by pancreatobiliary tract anomalies such as a choledochal cyst or abnormal union of the pancreatobiliary junction. Therefore, ERCP should be performed in pancreatitis of unknown cause. MRCP imaging has also improved and is useful in searching for a cause of acute pancreatitis in children[59]. In particular, MRCP should be performed before ERCP to detect any pancreatobiliary tract disease in children with initial onset of acute pancreatitis of unknown cause. However, in younger children, abnormal union of the pancreatobiliary junction is often difficult to delineate[21].

Severity assessment

Rapid and accurate assessment of severity is useful for selecting appropriate initial treatment and predicting the prognosis. In 2002, DeBanto *et al*[1] were the first to suggest a scoring system for predicting the severity of acute pancreatitis in children. This system is modified from the Ranson and Glasgow systems and consists of the following eight parameters: age (< 7 years old), weight (< 23 kg), white blood cell count at admission (> 18500 cells/μL), lactic dehydrogenase at admission (> 2000 U/L), 48-h trough Ca^{2+} (< 8.3 mg/dL), 48-h trough albumin (< 2.6 g/dL), 48-h fluid sequestration (> 75 mL/kg per 48 h), and 48-h rise in blood urea nitrogen (> 5 mg/dL). They set the cutoff for predicting a severe outcome at three criteria. However, this scoring system is not exact for Asian children[18]. Lautz *et al*[2] also reported that DeBanto pediatric scores have limited ability to predict acute pancreatitis severity in children and adolescents in the United States. Recently, we reported the usefulness of a new severity assessment that modified the acute pancreatitis severity scoring system of the Ministry of Health, Labour and Welfare of Japan (JPN score) for use in children[60,61]. The parameters of the pediatric JPN score were as follows: (1) base excess ≤ -3 mEq or shock (systolic blood pressure cutoffs according to age group); (2) PaO_2 ≤ 60 mmHg (room air) or respiratory failure; (3) blood urea nitrogen ≥ 40 mg/dL [or creatinine (Cr) ≥ 2.0 mg/dL] or oliguria (< 0.5 mL/kg per h); (4) lactate dehydrogenase ≥ 2 × the value of the upper limits; (5) platelet count ≤ $1 × 10^5/mm^3$; (6) calcium ≤ 7.5 mg/dL; (7) C-reactive protein ≥ 15 mg/dL; (8) number of positive measures in pediatric SIRS score ≥ 3; and (9) age < 7 years old or/and weight < 23 kg. The cutoff for predicting a severe outcome was set at three criteria.

The CT severity index has proven to be very useful in adults[62]. Recently, Lautz *et al*[58] also reported that the CT severity index was superior to a clinical scoring system for identifying children with acute pancreatitis at heightened risk for developing serious complications.

TREATMENT

The initial treatment for acute pancreatitis is to withhold oral intake of food or fluid to allow the pancreas to rest (*i.e.*, prevent stimulation of pancreatic exocrine secretions). Fluid and electrolyte supplementation, enzyme inhibition therapy, and treatment to relieve pain and

prevent infection are provided. It is important to gradually permit liquid and food intake at a suitable time while continuing treatment. This treatment strategy is based on a consensus conference and evidence accumulated in adult patients. The basic pathogenesis of acute pancreatitis does not greatly differ between adults and children, and the treatment selected for children should be similar to that in adults.

Infusion of extracellular fluid
Because fluid leaks into the surrounding tissue due to inflammation associated with acute pancreatitis, adequate infusion to supplement extracellular fluid is needed during initial treatment. In severe cases, increased vascular permeability and decreased colloid osmotic pressure causes extravasation of extracellular fluids into the surrounding tissue and retroperitoneum and then into the peritoneal cavity and pleural cavity, thus leading to large losses in circulating plasma volume[63]. This acute circulatory impairment causes a rapidly deteriorating condition in early acute pancreatitis.

DRUG THERAPY

Analgesics
Pain in acute pancreatitis is often intense and persistent, and pain control is required. Appropriate use of analgesics can effectively reduce pain, but this should not interfere with making a diagnosis or providing other treatments[64-66]. The analgesics used include pentazocine, metamizole, and morphine.

Antibiotics
In mild cases of acute pancreatitis, the incidence of infectious complications and mortality rates are low, and prophylactic antibiotics are usually not necessary. However, even in mild cases, antibiotics should be considered if severity increases or complications like cholangitis develop. In severe cases, antibiotics can reduce infectious pancreatitis complications and improve the prognosis[67]. Drugs should be selected with good tissue distribution to the pancreas.

Pancreatic protease inhibitors and octreotide
The Santorini Consensus Conference in 1997 concluded that gabexate mesilate did not contribute to reduced mortality rates in acute pancreatitis[68]. However, in severe acute pancreatitis, continuous infusion of large doses of gabexate mesilate may decrease complications and mortality rates[69]. Similar efficacy in children has been reported, but no clear evidence exists[70]. Protease inhibitors may be a part of combined modality therapy (especially to improve hemodynamic status), but judicious administration is advised in severe cases.

Octreotide was introduced in the early 1980s and offers several advantages over somatostatin, such as a much longer half-life and the option for either subcutaneous or intravenous administration[71]. Octreotide is a powerful inhibitor of exocrine pancreatic secretion and cholecystokinin production[72]. Several studies have evaluated the effect of octreotide on the incidence of clinical pancreatitis after ERCP and postoperative complications such as pancreatic duct fistula following pancreaticoduodenectomy and pancreatic transplantation[73,74]. Effectiveness in reducing complications in acute pancreatitis has not been demonstrated[75]. However, at the case report level, octreotide has been effective in treating pancreatic pseudocysts as a complication in acute pancreatitis and in preventing and treating drug-related pancreatitis due to ASNase, a key drug used to treat lymphocytic leukemia in children[76-78]. As a somatostatin derivative, the most common adverse effect of octreotide is abdominal distention, but adverse effects such as failure to thrive are unlikely if octreotide is given for only 2-6 wk.

NUTRITIONAL SUPPORT
In severe pancreatitis, the early initiation of enteral nutrition reduces the incidence of infections and leads to shorter hospital stays[79]. An enteral feeding tube is placed in the duodenum or in the jejunum past the ligament of Treitz[80]. This type of nutrition is recommended to reduce stimulation of exocrine pancreatic secretion.

Control of abdominal pain and serum pancreatic enzyme levels should be considered in deciding when to resume oral intake. If serum pancreatic enzymes are decreasing, overall status is good, and abdominal pain has subsided, liquid intake can be started. If serum amylase and lipase levels are approximately less than two times the upper normal limits, a fat-restricted diet should be started[81]. Energy and fat intake can gradually be increased with careful monitoring.

Specific treatment for severe pancreatitis
In patients with infected pancreatic necrosis, surgical drainage and pancreatectomy may be indicated. Specific treatments such as continuous hemodiafiltration to remove humoral mediators and continuous regional arterial infusion of a protease inhibitor and antibiotics have been effective in adults[82,83]. These specific treatments have also been effective and lifesaving in children[84,85]. Although there is no universally acceptable scoring system for predicting the severity of childhood acute pancreatitis, consideration should be given to early transfer of severe patients to a medical center where intensive treatment is available.

Endoscopic treatment and surgery
Anatomic anomalies such as abnormal union of the pancreatobiliary junction are an indication for surgery. In patients with outflow tract obstruction of pancreatic juices caused by ampulla of Vater anomalies or pancreatic divisum, endoscopic sphincterotomy is effective.

Infectious complications should be clinically suspected if fever or signs of inflammation recur during the course of acute pancreatitis. Symptoms often become

prominent 2 wk or more after the onset of pancreatitis. The definitive diagnosis of infected pancreatic necrosis can be made by CT- or ultrasound-guided local fine-needle aspiration and bacteriologic cultures[86,87]. However, this procedure may be difficult in children. Therefore, worsening blood test results, positive blood cultures, positive blood endotoxins, elevated serum procalcitonin levels, and CT findings of the pancreas may serve as clues to a diagnosis of infected pancreatic necrosis[88].

Patients whose general condition is stable can be conservatively treated with antibiotics and observed, but if their condition does not improve, a necrosectomy is required. Necrosectomy early in pancreatitis is associated with a high mortality rate, so it should ideally be performed after the patient's hemodynamic status and general condition have stabilized[89]. Percutaneous necrosectomy, endoscopic transgastric necrosectomy and laparoscopic pancreatic necrosectomy have recently been reported as less invasive treatments in adults and a few children[90-92]. Pancreatic abscesses generally require percutaneous, endoscopic, or surgical drainage.

Pancreatic pseudocysts are cysts that develop due to injury of the pancreatic duct and extravasation of fluid. These occur 4 wk or later after the onset of pancreatitis. Treatment is indicated for pseudocysts if their size does not decrease, if they are accompanied by abdominal pain, or if there are complications of infection or hemorrhage. Endoscopic ultrasound-guided transgastric puncture and drainage can safely be performed in these cases[93,94].

CONCLUSION

Currently, our approach to acute pancreatitis in children mainly depends on physician experience and knowledge gained from acute pancreatitis in adults. Acute pancreatitis in children tends to be considered a difficult disease, even by pediatric gastroenterologists. However, with recent advances in diagnostic techniques and treatment methods, unfamiliar and difficult diseases are becoming controllable diseases once they are better understood. In order to improve treatment outcomes in patients with childhood acute pancreatitis, future studies focusing on developing a scoring system for predicting the severity of acute pancreatitis and identifying the potential effective treatment modalities for children should be conducted.

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Genetics of acute and chronic pancreatitis: An update

VV Ravi Kanth, D Nageshwar Reddy

VV Ravi Kanth, Asian Healthcare Foundation, Somajiguda, Hyderabad 500082, Andhra Pradesh, India

D Nageshwar Reddy, Asian Institute of Gastroenterology and Asian Healthcare Foundation, Somajiguda, Hyderabad 500082, India

Author contributions: Ravi Kanth VV wrote the initial draft; Nageshwar Reddy D reviewed the manuscript and approved the final draft.

Correspondence to: Dr. D Nageshwar Reddy, Director, Asian Institute of Gastroenterology and Asian Healthcare Foundation, 6-3-661, Somajiguda, Hyderabad 500082, India. aigindia@yahoo.co.in

Telephone: +91-40-23378888 Fax: +91-40-23324255

Abstract

Progress made in identifying the genetic susceptibility underlying acute and chronic pancreatitis has benefitted the clinicians in understanding the pathogenesis of the disease in a better way. The identification of mutations in cationic trypsinogen gene (*PRSS1* gene; functional gain mutations) and serine protease inhibitor kazal type 1 (*SPINK1* gene; functional loss mutations) and other potential susceptibility factors in genes that play an important role in the pancreatic secretory functions or response to inflammation during pancreatic injury has changed the current concepts and understanding of a complex multifactorial disease like pancreatitis. An individual's susceptibility to the disease is governed by genetic factors in combination with environmental factors. Candidate gene and genetic linkage studies have identified polymorphisms in cationic trypsinogen (*PRSS1*), *SPINK1*, cystic fibrosis trans-membrane conductance regulator (*CFTR*), Chymotrypsinogen C (*CTRC*), Cathepsin B (*CTSB*) and calcium sensing receptor (*CASR*). Individuals with polymorphisms in the mentioned genes and other as yet identified genes are at an enhanced risk for the disease. Recently, polymorphisms in genes other than those involved in "intra-pancreatic trypsin regulatory mechanism" namely Claudin-2 (*CLDN2*) and Carboxypeptidase A1 (*CPA1*) gene have also been identified for their association with pancreatitis. With ever growing number of studies trying to identify the genetic susceptibility in the form of single nucleotide polymorphisms, this review is an attempt to compile the available information on the topic.

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Key words: Chronic pancreatitis; Acute pancreatitis; Genetic susceptibility; Single nucleotide polymorphisms; Inflammation

Core tip: Pancreatitis is a progressive inflammatory disease. Though the pancreas has adequate protection against environmental and metabolic stress, if the magnitude of this stress exceeds the threshold which the organ can handle, it leads to pathologic effects. Although genetic variables have been identified that affect the function of pancreas, namely polymorphisms in serine protease inhibitor kazal type 1 (*SPINK1*), polymorphisms in cationic trypsinogen (*PRSS1*) and Chymotrypsinogen C (*CTRC*) genes in the acinar cells and cystic fibrosis trans-membrane conductance regulator (*CFTR*), calcium sensing receptor (*CASR* genes in the ductal cells leading to pancreatitis, off late many genetic factors outside of the "intra-pancreatic trypsin regulatory mechanism" have been identified for their role in pancreatitis. This review is an update on the genetic aspects of acute and chronic pancreatitis.

INTRODUCTION

Chronic pancreatitis (CP) is a disease associated with

inflammation where the secretory parenchyma of the pancreas is progressively destroyed. There is involvement of several known risk factors and processes such as inflammation, necrosis, apoptosis or duct obstruction despite the heterogeneity in pathogenesis. The process of fibrosis usually leads to progressive worsening in lobular morphology, structure of pancreas, changes in arrangement and composition of the islets and deformation of the large ducts[1]. These conditions lead to diabetes that is due to irreversible morphological and structural changes and exocrine and endocrine dysfunction[2]. The major types of pancreatitis are acute pancreatitis (AP), recurrent acute pancreatitis (RAP) and CP.

In spite of an individual carrying a genetic risk and being subjected to oxidative or metabolic stress, the pancreas is histologically normal in appearance in the pre-acute phase. "First hit" in terms of injury due to excess alcohol consumption, metabolic factors, hyperlipidemia, gallstones and genetic factors leads to AP-which is a sentinel AP event (SAPE)[3]. During this proinflammatory phase, inflammatory related damage occurs due to the infiltration of the pancreas with inflammatory cells. This phase may end through an anti-inflammatory response that is mediated partly by tissue macrophages and is associated with the activation of stellate cells and subsequent proliferation causing fibrosis. However clinical recovery is attained in most of the cases.

If this phase is followed by RAP due to genetic risks namely polymorphisms in serine protease inhibitor kazal type 1 *(SPINK1),* polymorphisms in cationic trypsinogen *(PRSS1)*, cystic fibrosis trans-membrane conductance regulator *(CFTR)* genes and other as yet unknown genes) or chronic cell stressors develop like alcohol, smoking, oxidative stress, *etc.*, after the SAPE (second hit), it leads to CP which is due to chronic inflammation and progressive fibrosis. CP may also manifest as a direct result of extensive pancreatic necrosis, duct obstruction in the proximal region directly resulting from severe AP which is independent and without the second hit[4].

Many risk factors that contribute varyingly to pancreatitis have been identified. These include alcohol, metabolic factors, toxins, insecticides, certain medications, viral and bacterial infections, trauma caused by surgery[5]. Growing evidence suggests a substantial contribution of genetic predisposition to pancreatitis. As early as 1950's, genetic studies on pancreatitis suggested that it may be an inherited disease[6]. After this initial description, a mutation inherited in autosomal dominant mode was identified in the cationic trypsinogen gene that is located on 7th chromosome in individuals with hereditary pancreatitis[7,8]. Further to this, a number of other mutations/polymorphisms in genes that have a role in inhibition, regulation or modulation of the pancreatic trypsin activity, secretory function and inflammatory injury respectively were identified. Mutations in the *PRSS1, SPINK1, CFTR* and polymorphisms in other genes namely the ones regulating the response to inflammation [tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-10][9] are

the major genetic contributors to the development of AP and CP.

A model (two hit model) for the pathogenesis of pancreatitis has been proposed[10], suggesting that "there is a loss of balance between events associated with activation and degradation of active trypsin enzyme leading to the presence of persistent "super-trypsin" with in the acinar cell that is due to mutations or polymorphisms in genes namely *SPINK1,* Cathepsin B *(CTSB)*, Chymotrypsinogen C *(CTRC)* and other yet to be identified susceptibility genes. This loss of balance leads to inflammation and these events are the first hits that contribute to the pathogenesis of pancreatitis". The presence of additional genetic and/or environmental risks leading to one or more phenotypes namely fibrosis, stone formation and/or diabetes and these events are the second hit.

AP: DEFINITION, SYMPTOMS AND RISK FACTORS

AP is a syndrome of acute and sudden inflammation of the pancreas. Clinically, it is detected by upper abdominal pain with sudden onset, digestive enzymes namely pancreatic amylase and lipase that are elevated in the serum and/or typical findings like edema, peripancreatic fat stranding, fluid collection on the abdominal imaging studies. The process in AP is initiated by an injury that is acute followed by an inflammatory response (also acute) which is mostly out of proportion and to the extent of tissue injury. The above response is due to premature activation of digestive enzymes in the pancreas that digest the tissue, consequently activating the inflammatory cascade. The immune system may also be cross-activated by the activated pancreatic digestive enzymes. Many risk factors for AP have been identified. The most important of them being duct obstruction by gall stones, parasites, tumors, anatomical abnormalities and endoscopic retrograde cholangio-pancreatography; metabolic factors like hyperlipidemia, hypercalcemia and acidosis; toxins like ethyl alcohol, insecticides, scorpion toxins, medications (azathioprine, NSAIDs, tetracycline, *etc.*); Bacterial and viral infections, trauma caused by blunt or penetrating or surgery apart from genetic susceptibility namely mutations in *PRSS1, SPINK1* and *CFTR*[5].

CP: DEFINITION, SYMPTOMS AND RISK FACTORS

CP is a disease associated with inflammation that is progressive and is characterized by three main features. Abdominal pain that is recurrent or persisting at the clinical level, damage of the parenchyma in pancreas with irregular sclerosis and inflammation, accompanied by ductal dilation, strictures or stones at the morphological level and finally a progressive loss of exocrine and endocrine functions at the functional level[11-13]. Based on the etiologies and risk factors, a working classification for CP

Table 1	General genetic information of the genes which confer susceptibility to pancreatitis						
Name of the gene	Upstream gene variants	Downstream gene variants	Non-coding exon variants	Synonymous variants	Missense variants	Stop gained	Intron variants
CTRC	490	430	102	28	57	5	789
CASR	580	732	129	433	1459	57	4707
PRSS1	1031	1634	431	126	280	6	637
CTSB	5763	11413	621	682	1261	10	18675
SPINK1	366	252	38	8	37	0	236
CFTR	1193	2377	87	447	2533	558	13723
CLDN2	205	171	0	36	78	0	560

CTRC: Chymotrypsin C; *CASR*: Calcium sensing Receptor; *PRSS1*: Trypsinogen Gene; *CTSB*: Cathepsin B; *SPINK1*: Serine protease inhibitor kazal type 1; *CFTR*: Cystic fibrosis transmembrane conductance regulator; *CLDN2*: Claudin 2.

Table 2	Summary[1] of the polymorphisms in genes related to pancreatitis			
Name of the gene	Chromosome	No. of splice variants	Length (bp) of exon region	No. of exons
CTRC	1	4	898	8
CASR	3	4	5009	7
PRSS1	7	6	800	5
CTSB	8	35	3875	10
SPINK1	5	3	542	4
CFTR	7	11	6128	27
CLDN2	X	3	3150	2

[1]Extracted from ENSEMBL. Upstream Gene variants: A sequence variant located 5′ of a gene. Downstream gene variants: A sequence variant located 3′ of a gene. Non-coding exon variants: A sequence variant that changes non-coding exon sequence. Synonymous variants: There is no change in the resulting aminoacid. Missense variants: Variant that changes one or more bases, resulting in a different aminoacid but where the length is preserved. Stop gained: Sequence variant whereby at least one base of a codon is changed, resulting in premature stop codon, leading to a shortened transcript. Intron variants: a variant occurring within an intron. *CTRC*: Chymotrypsin C; *CASR*: Calcium sensing Receptor; *PRSS1*: Trypsinogen Gene; *CTSB*: Cathepsin B; *SPINK1*: Serine protease inhibitor kazal type 1; *CFTR*: Cystic fibrosis transmembrane conductance regulator; *CLDN2*: Claudin 2.

has been elaborated by the American Gastroenterological Association according to its prevalence and mechanism named TIGAR-O classification system (toxic-metabolic, idiopathic, genetic, autoimmune, recurrent and severe AP, obstruction)[14]. The toxic metabolic include alcohol, smoking (tobacco), hyperlipidemia, hypercalcemia, chronic renal failure and certain medications; idiopathic includes early onset, late onset and tropical; mutations in cationic *PRSS1* gene, *CFTR* gene, *SPINK1*, α-1 antitrypsin deficiency and other unidentified genes comprise genetic risk; autoimmune includes isolated autoimmune chronic pancreatitis, autoimmune syndromic CP including Sjogren's syndrome-associated CP, primary biliary cirrhosis-associated CP and inflammatory bowel disease-associated CP. Recurrent and severe AP-associated CP includes post necrotic (severe AP), vascular disease/ischemic and post-irradiation. Obstructible risk factors include sphincter of Oddi disorders, pancreas divisum, duct obstruction (tumor), preampullary duodenal wall cysts and post-traumatic pancreatic duct scars.

GENETIC RISK FACTORS FOR ACUTE AND CP

It has long been suggested that inappropriate activation of trypsinogen in the pancreas is the first and most important step in the development of pancreatitis[15] and all the known genetic susceptibility factors for pancreatitis identified till date can be categorized as members of the intra-pancreatic trypsin regulatory mechanism and were identified employing a candidate-gene approach based on the above mechanism and they include polymorphisms/mutations in genes namely *CTRC*, *CASR*, Trypsinogen gene (*PRSS1, 2* and *3*), Cathepsin B (*CTSB*), *SPINK1/PST1*, *CFTR* gene. General information about the genes is presented in Table 1. A recent study[16] identified an underlying genetic susceptibility in approximately half of idiopathic CP patients, when they screened for mutations in *PRSS1, SPINK1, CTRC* and *CFTR* genes, emphasizing the important role of genetics in CP. A detailed list of different types of polymorphisms identified in these genes till date has been extracted from ENSEMBL and presented in Table 2 and the list of polymorphisms in these genes are also listed in the web site www.pacreasgenetics.org, however only the important polymorphisms/mutations have been discussed in detail in this review.

Trypsinogen (PRSS1, 2 and 3) genes

PRSS1, anionic trypsinogen (*PRSS2*) and mesotrypsinogen (*PRSS3*) are the three types of trypsinogen that are expressed by the pancreas to an extent of two-thirds to one-third to less than 5% respectively[17,18]. Eight trypsinogen genes are shown to be located in the beta T-cell receptor locus at 7q35[19]. The *PRSS1* gene that is mapped to the long arm of chromosome 7 encodes the trypsin-1 (TRY-1) protein[8,20]. Important mutations (gain of function namely A16V, N29I, R122H) have been identified in the *PRSS1* gene that are associated with hereditary pancreatitis in Caucasians[21,22], French[23], D162D variant in Chinese[24] however a study from India reported that *PRSS1* gene mutations are not associated with CP[25]. A study from Korea reported that 5.4% of subjects with idiopathic CP and 40% with pancreatitis that is hereditary carried R122H mutation in the *PRSS1* gene and other variants were not reported apart from R122H. None

of the 50 controls had the mutation[26]. One important study[27] screened for *PRSS1* mutations in a Belgian patient with sporadic CP and observed a migration pattern that is altered different from the transition (g.133283G > A) in exon 3 of the gene. Subsequent analysis by DNA sequencing revealed a DNA variant that was novel (g.133283-133284GC > AT) also resulting in R122H, however they concluded that in contrast to the change in codon CGC to CAC, codon CGC > CAT strongly suggested an alternative mutational mechanism of gene conversion.

Apart from the polymorphisms and their associations with pancreatitis, studies have also looked in to the copy number variations (CNVs) for their role in pancreatitis. A study[28] identified a duplication and triplication of 605kb segment on chromosome 7q35 in French ICP patients, which increased the copy number of *PRSS1* and 2 genes that code for anionic trypsinogen. The same study identified a trypsinogen gene that was hybrid with exon 1, 2 from *PRSS2* and exons 3 to 5 from *PRSS1*, which had two gain of function effects namely increase in trypsinogen gene copy number with N29I mutation in it. The 605kb segment duplication was also assessed further in French and Indian patients with idiopathic CP (ICP) and concluded that it was associated with French ICP but not in Indian patients with CP[29], however the CNVs in *PRSS3* were not associated[30].

Serine protease inhibitor Kazal type 1/pancreatic secretory trypsin inhibitor gene

SPINK 1/pancreatic secretory trypsin inhibitor (*PSTI*) is a specific trypsin inhibitor and an acute phase protein which is secreted by the acinar cells[31]. The gene encoding *SPINK1* has 4 exons and 3 introns that is located at 5q32 and is approximately 7.5kb long[32]. SPINK1 protein plays a role in the prevention of premature activation of zymogen that is catalyzed by trypsin within the pancreatic duct system or the acinar tissue. A reactive site in the protein serves as a specific target substrate for trypsin[33] and it can inhibit up to 20% of the activity of pancreatic trypsin. It is the first line of defense against auto digestion, thereby protecting the pancreas[9], however inhibition of trypsin by *SPINK1* is temporary as trypsin may target the trypsin-SPINK1 complex and subsequently degrade the inhibitory molecule and restore trypsin activity[34]. *SPINK1* mutations cause a loss of function mutations as against *PRSS1* which generate gain of function mutations. There are several mutations/polymorphisms that are identified till date in the *SPINK1* gene (Table 2), however N34S is the most common missense mutation, that is a substitution of asparagine by serine at codon 34. N34S polymorphism was found in individuals especially without a family history and many studies have confirmed its association in different ethnic groups[25,35-37]. A substantial number of patients (15%-40%) with ICP carry N34S mutation in either heterozygous or homozygous state based on the above studies. The *SPINK1* polymorphisms (N34S) are in complete linkage disequilibrium

with other variants that are located in the introns[38]. Other mutations/polymorphisms have also been identified namely a promoter mutation (-215-A and -215 G > T), a mutation in the start codon that destroys the only translational initiation codon of *SPINK1* (2 T-C, Met to Thr; MTT)[39], -53C > T; -41G > A, -2C > A; L14P; D50E; IVS3 + 125C > A; IVS3 + 184T > A; R65Q; R67C which were reported predominantly in single patients or families[35,38,40].

Polymorphisms in *SPINK1* gene are generally associated with loss of function. Although the *SPINK1* N34S polymorphism is associated with pancreatitis, the association is weak with very few individuals with the mutation developing pancreatitis some time during their life time[35,41]. Furthermore there is no difference in the severity of the disease with respect to the heterozygous and homozygous genotypes of SPINK1; there are complex interactions and the effect of the mutation depends on the reduction in the enzyme. Pancreatitis may be initiated in the homozygous N34S state, however the heterozygous genotype may only cause a lowering of the enzyme level and it requires other additional factors (genetic and environmental) to initiate the disease[42]. Therefore in general *SPINK1* polymorphism is hypothesized to be a susceptibility factor for a polygenic complex trait or a disease modifier[3] with polymorphisms in other genes being involved.

Apart from the above polymorphisms, two copy number mutations (deletions) in the SPINK1 gene that were associated with loss of function and encoding pancreatic secretory trypsin inhibitor (*PSTI*) were identified by a study[38]. In a particular family these deletions were co-inherited with a missense mutation (p.L997F) in the *CFTR* gene, suggesting complex interactions between the CNVs and single nucleotide substitutions contributing to the disease phenotype. SPINK1 polymorphisms are common in the general population (approximately 2%) but are shown to be significantly associated with pancreatitis.

Chymotrypsin C gene

CTRC encodes Chymotrypsin C, a digestive enzyme. It is produced by the acinar cells in the pancreas. It is packaged with zymogen granules and is secreted along with other digestive enzymes from the pancreas. Prematurely activated trypsin is destroyed by CTRC by acting on the molecule within the calcium-binding loop in the absence of calcium and therefore is a crucial candidate gene in the pathogenesis of pancreatitis[43]. Many polymorphisms have been identified in this gene till date (Table 2). A study[44] had sequenced all the 8 exons (8.2 kb) of the *CTRC* gene in a total of 621 individuals with idiopathic or hereditary CP and 614 control subjects of German origin and identified that the large majority of the variants were in 2nd, 3rd and 7th exons. Only exons 2, 3 and 7 were sequenced in an additional 280 CP patients and 2075 controls for exons 2 and 3 and 2190 controls for exons 7. Although a number of missense and deletion variants were found they concluded that the two most frequent variants

which were significantly overrepresented in the pancreatitis group as compared to the controls were c.760C > T (p.R254W) and c.738_761del24 (p.K247_R254del) (30/901 (3.3%) affected individuals but only in 21/2804 (0.7%) controls], both of which were located in exon 7. Furthermore, this group also studied 71 and 84 individuals of Indian origin with tropical pancreatitis and controls respectively, and suggested a higher frequency of *CTRC* alterations in this cohort [10/71 (14.1%) in Tropical pancreatitis Vs 1/84 (1.2%) controls] as compared to the German cohort and two relatively frequent variants were found in the Indian cohort namely c.217G > A (p.A73T) missense alteration and the c.190_193del ATTG (p.I64LfsX69) frame shift deletion[44]. Another study from India[45] identified 14 variants in 584 CP patients and 598 normal subjects [71/584 CP patients (12.2%) and 22/598 controls (3.7%)], when all the eight exons and flanking regions of the *CTRC* gene were sequenced. It was p.V235I variant which was common in the Indian CP patients as against the p.K247_R254del variant in the Caucasians. Apart from this variant the study also identified other pathogenic variants namely p.A73T and c.180C > T as significantly associated with Indian CP.

Cathepsin B gene

The human *CTSB* is 25.6kb. It has 12 exons. Several transcript species are known to be produced by alternative splicing[46]. It is hypothesized that chronic pancreatitis is a result of mutations in the *CTSB* gene and they may be involved in premature activation of trypsinogen or inappropriate localization[47]. A study on the CTSB gene polymorphisms and tropic calcific pancreatitis identified significant association of Val26Val polymorphism (allele frequency of 0.48 in patients *vs* 0.30 in controls) with Odds of 2.15 apart from differences in the mutant allele frequencies that are significant at Ser53Gly (allele frequency of 0.10 *vs* 0.04 in patients and controls respectively) and C595T SNPs (allele frequency of 0.12 *vs* 0.20 in patients and controls respectively. Further L26V polymorphism was equally as common in N34S positive and wild type patients suggesting that CTSB is involved independently with the disease. This study suggested that *CTSB* polymorphisms may be associated with pancreatitis more so in the absence of mutations in *PRSS1* gene and N34S *SPINK1* polymorphism proposed to play a disease modifier role[47], however another study failed to associate polymorphisms in this gene with pancreatitis in European cohort (allele frequency of 0.398 in patients and 0.48 in cotrols)[48].

Calcium-sensing receptor gene

Auto-activation and autolysis are processes in which trypsinogen molecule is activated to trypsin and is also degraded by other trypsin molecules. For the mentioned purpose, two specific cleavage sites exist for potential attack by other trypsin molecules. Lysine 23 (L23) is the first site and arginine 1122 (R122) the second. The cleavage of L23 causes trypsinogen activation to trypsin

with 8-amino acid trypsinogen activation peptide being released while R122 cleavage causes inactivation of trypsin. The susceptibility of the two sites for an attack is regulated by calcium concentration and concentration dependent occupation of the calcium binding sites[49]. In normal acinar cells low calcium concentrations are prevalent and these low concentrations limit the activation of trypsinogen, thereby promoting inactivation of trypsin by exposing the second site (R122), however calcium hyper stimulation or dysregulation in the acinar cells favors activation of trypsinogen and prevention of trypsin inactivation[50]. Thus regulation of calcium levels (intra-acinar) is critical for preventing trypsinogen activation and pancreatic injury. CASR plays a major and important role in maintaining the calcium homeostasis through its effect on renal tubules and parathyroid gland. A variety of hypercalcemia-associated syndromes are associated with genetic variants in the *CASR* gene[51]. The first of the reports associating *CASR* mutations with CP came from a family study of 5 individuals who were all heterozygous for the N34S *SPINK1* polymorphism. Only two of the 5 heterozygous individuals developed CP and both these individuals presented with a T > C mutation at position 518 in the *CASR* gene, that is a leucine to proline amino acid change in the extracellular domain of the CASR protein[52], suggesting that *CASR* mutations may be a predisposing genetic factor that may increase the susceptibility for CP. Another study[53] that screened for mutations in *SPINK1* and *CASR* gene on a small Indian cohort of 35 patients with Tropical chronic pancreatitis (TCP) and an equal number of controls reported that a combination of mutations in both the genes was seen in 6% of the patients, while 22% had mutation in single gene, suggesting that *CASR* mutations may be a risk for TCP and that risk may be further increased with associated *SPINK1* mutation. A study by Muddana *et al*[54] initially included 115 subjects with pancreatitis and 66 controls. Of the study group, 57 patients and 21 controls were predetermined to carry the N34S *SPINK1* polymorphism. Based on the initial results, the study included an additional 223 patients and 239 controls to analyze the three common non-synonymous SNPs in exon 7 that were found to be significant from the initial study. The *CASR* exon 7 polymorphism (R990G) was significantly (Odds, 2.01 and *P* = 0.01) associated with CP and the association of this SNP was stronger in subjects with moderate to heavy alcohol consumption. This study however did not find any significant associations between the various *CASR* genotypes and *SPINK1* N34S in CP. None of the earlier reported polymorphisms from Germany and India were also detected in this US-based study. All the association studies suggest that recurrent trypsin activation/dysregulated calcium and failed inhibition increase the risk of pancreatitis *via* the intracellular calcium dysregulation.

CFTR gene

The impact of *CFTR* gene continues to be debated, although variants in this gene are strongly associated

with pancreatitis. *CFTR* gene in humans has 27 exons, is located at 7q31 and is 250 kb in length[55]. For the proper functioning of the duct cells in the pancreas and other anion secreting epithelial cells, CFTR anion channel is a critical molecule. CFTR apart from regulating the functions of other channels also conducts both chloride and bicarbonate channels, the opening and closing of which controls the bulk of fluid secretion from the pancreas[50]. The association between idiopathic CP and *CFTR* mutations was demonstrated in 1998[56,57]. More than 1200 mutations have been identified and based on the mechanism by which they disrupt the function; they are classified in to five different groups with group V mutations subsequently being included in group I (as they cause functional alterations in the levels of mRNA)[58]. Class I mutations affects biosynthesis, class II mutations affect protein maturation, class III affect chloride channel regulation/gating while class IV mutations affect chloride conductance[59]. An additional class of mutations was proposed by Haardt *et al*[60] as class VI which included protein stability mutations.

A higher frequency of mutations in the *CFTR* gene was seen in a significant number of patients (30%) with ICP. There was six and two times higher frequency of CFTR mutations and 5T allele respectively in patients[56,57,61]. With few of these mutations there was a reduction in the amount of functional CFTR. The others might be a combination of a severe and a mild mutation or either type of mutations with 5T allele in intron 8 of the gene[9]. There is an increased risk (up to 40 fold) for pancreatitis when individuals are compound heterozygotes[62]. Complete coding sequences of the *CFTR*, P*RSS1* and *SPINK1* genes were analyzed for mutations and it was seen that 25%-30% of the patients with CP carried at least a single mutation in the *CFTR* gene and majority were compound heterozygotes for a *CFTR* mutation or were trans-heterozygotes for *CFTR, PRSS1* and *SPINK1* mutations[62,63]. Furthermore, a combination of two *CFTR* mutations and N34S in *SPINK1* gene increases the risk of pancreatitis by 900 fold[9]. It is clear from these studies that *CFTR* variants are associated with CP, however the mechanisms of the complex interactions of various susceptibility loci has to be understood in a better way.

Proinflammatory cytokine genes

It is already established that the cytokine profile within the pancreas is different in CP as compared to normal pancreas[64]. A potential factor that could affect the production of proinflammatory cytokines are polymorphisms in these genes. Association studies involving polymorphisms in various cytokine genes have shown varying results in various populations. Various genes namely *TNF-a* (tumor necrosis factor-α), *Monocyte chemoattractant protein-1*, and *IL-8*[65-67] have been studied for their association with pancreatitis.

It is known that *TNF-a* along with *IL-1* is a major early cytokine to mediate the systemic inflammatory response syndrome (SIRS)[68-70]. A study[71] reported the

association between *TNF-a* -238 AG but not -308 SNP genotype with organ failure (shock and/or respiratory failure) and in the IL-6 gene the CC genotype at position 174 was associated with biliary etiology of AP. The study included 84 patients with AP (no controls were included) and known polymorphisms in *TNF-a*, interleukin 1 (*IL-1*), IL-1 receptor antagonist (*IL1RN, IL-6* and *IL-10)* were genotyped for etiology associated susceptibility and severity, however other polymorphisms like *TNF-a*-1031, -863 and -857 SNPs were not included in the study. Another study[72] reported a negative association between *TNF-a*-308 and severity of pancreatitis (397 patients and 300 controls with major allele frequency in *TNF* gene being 0.87 for patients with AP and 0.86 for controls) from Finland, however they did not study the *TNF-a*-238 SNP. These results were similar to studies reported from United Kingdom, by[73], who studied 190 and 102 AP patients and controls respectively and Sargen *et al*[74], who studied 135 AP and and 107 controls repesctively (78.3% and 84.4% for *TNF-a*-308 and 21.7% and 15.6% for *TNF-a*-238 in controls and AP repetively). However, *TNF-a*-308 allele was reported to be associated with severe AP in Hungarian patients[75]. The study included 77 patients (mixed etiology and grouped according to the severity of the disease on the basis of Ranson scores) and 71 controls. Another study[76] associated *TNF-a*-308 allele with shock in patients with severe AP, however suggested that the polymorphism played no part in disease severity or susceptibility. The study included 208 AP cases and 116 ethnicity matched controls. A recent meta-analysis[77] integrated the previous findings on *TNF-a*-308 G > A and -238 G > A alleles and explored whether the polymorphisms were associated with susceptibility and severity to pancreatitis. The study included 1569 pancreatitis cases and 1330 controls from 12 published case-control studies and concluded that polymorphisms in these two sites did not alter the risk of pancreatitis.

Monocyte chemoattractant protein 1 (MCP-1) is a member of the C-C chemokine family. It exerts a strong chemo attractant activity in macrophages, lymphocytes and monocytes[78]. A common polymorphism-2518 A > G alters the expression of the gene with G allele being associated with higher levels of MCP-1 protein which is associated with higher risk of pancreatitis. A study from United States[65] included 77 consecutive patients and 116 controls for the mentioned genotype and concluded that the -2518 genotype is a risk factor for severe AP (12 of 14; 86% with AP *vs* 50 of 116; 43% control subjects) and also suggested that MCP-1 serum levels appear to be an accurate predictor of severity of AP and death when measured early in the course of the disease. Another study from Italy[79] studied 118 AP, 64 ARP, and 142 CP patients and 88 controls and concluded that all patients with pancreatic inflammatory disease had significantly higher serum MCP-1 levels. A study[80] studied the relationship between a polymorphism in the *MCP-1* gene (-2518A/G) and AP in the Han population of Suzhou, China and suggested an increased risk of AP associated

with G allele [72.4% (113/156) and 76.1% (35/46) in severe AP; 47.1% (113/240)]. However, the 2518A/G polymorphism in the *MCP-1* gene did not significantly alter the susceptibility to CP[81].

Interleukins are proinflammatory cytokines and polymorphisms in these genes have been shown to affect the immune response[82]. A meta-analysis[83] on the inteleukin gene polymorphisms which included a total of 10 studies, covering a total of 1220 AP cases and 1351 controls showed evidence for significant association between *IL-8* -251 T/A (rs4073) polymorphism and AP risk, suggesting that *IL-8* -251 A allele was associated with an increased risk of AP. However, there were no significant associations between *IL-1β* [IL-1β +3954 C/T (rs1143634) and IL-1β -511 C/T (rs16944)], IL-6 [IL-6 -174 G/C (rs1800795) and *IL-6* -634 C/G (rs1800796)] and *IL-10* [IL-10 -1082 A/G (rs1800896), *IL-10* -819 C/T (rs1800871) and *IL-10* -592 C/A (rs1800872)] gene polymorphisms and AP risk. In summary, the study concluded that the IL-8 -251 T/A polymorphism was associated with an increased risk of AP. In addition, there were no significant associations between IL-1β, IL-6 and IL-10 gene polymorphisms and AP risk.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine. It is released by macrophages and lymphocytes[84]. It plays an important pathogenic role in AP and a study[85] investigated the role of -173 G > C polymorphism and the (CATT) n repeat microsatellite at position -794 in 164 patients with AP and 197 controls C allele 58/160 [18.1% in AP *vs* 47/097 (11.9%) in controls]. There was no significant difference in the repeat length of the microsatellite marker between patients and controls, however the C allele of the -173 G > C genotype was significantly higher in patients.

Claudin-2 and Carboxypeptidase A1 gene

New susceptibility loci for CP have been identified. The first SNP in the Claudin-2 (*CLDN2*) locus is the outcome of the first and only reported Genome wide association study for pancreatitis till date, which included 1676 cases and 4507 controls in stage Ⅰ and 910 cases and 4170 controls in stage Ⅱ. The study identified two SNPs namely one SNP in *PRSS1-PRSS2* locus (allele frequency of 0.576 in controls *vs* 0.634 in pancreatitis) and the other in the Claudin-2 locus (*CLDN2*) (allele frequency of 0.261 in controls *vs* 0.322 in pancreatitis). The SNP in the *PRSS1* locus affects susceptibility by altering the expression of trypsinogen and the SNP in the *CLDN2* is associated with atypical localization of claudin-2 in pancreatic acinar cells. Homozygous or hemizyogus genotype (in females and males) confers the greatest risk and the alleles also interact with alcohol consumption to increase the risk of pancreatitis[86]. Another study[87] analyzed variants in Carboxypeptidase A1 (*CPA1*) encoding carboxypeptidase A1, primarily in Germany discover set and in thee replication sets from Europe, India and Japan. *CPA1* variants were associated with non-alcoholic CP with varying levels of significance in the discovery [29/944 (3.1%) of Ger-

man cases and 5/3,938 (0.1%) controls] as well as all the three replication sets 8/600 (1.3%) of European cases and 9/2,432 (0.4%) controls, 5/230 (2.2%) of Indian cases and 0/264 controls and 5/247 (2.0%) of Japanese cases and 0/341 controls. The study concluded that variants may confer an increased risk of CP and the mechanism may involve endoplasmic reticulum stress that may be induced by misfolding rather than trypsin activity that is elevated.

GENETIC TESTING FOR *PRSS1*, *SPINK1* AND *CFTR* GENES - WHEN TO ORDER THE TEST?

A valuable diagnostic genetic test to investigate acute and CP has been added ever since a point mutation in the *PRSS1* gene has been identified. Consensus guidelines for ethical molecular genetic testing for hereditary pancreatitis has been proposed[88] which recommends it under the following conditions: (1) Unexplained two or more (recurrent) episodes of documented pain that are separate with hyoeramylasemia attack; (2) Idiopathic CP; (3) Family history of pancreatitis [in a parent, sib, child (first degree) and in aunt, uncle or grand parent (second degree)]; (4) A need to exclude significant concern of hereditary pancreatitis in a child with an unexplained episode of documented pancreatitis that required a hospitalization; (5) As part of research protocol that is approved. Genetic testing (*PRSS1* mutations) in children below 16 years is indicated after; (6) Hospitalization that was required in an individual because of an episode of documented pancreatitis of unknown etiology that is severe enough; (7) Pancreatitis of unknown etiology in an individual with two or more documented episodes; (8) A child with an episode of documented pancreatitis, who has a relative with hereditary pancreatitis mutation that is known; (9) Recurrent abdominal pain (unknown etiology) in a child, where there is a distinct clinical possibility of hereditary pancreatitis; and (10) Diagnosis of hereditary pancreatitis as a distinct clinical possibility in an individual with CP of unknown etiology[88].

Currently genetic testing for mutations in SPINK1 or CFTR genes is considered as premature as the identification of mutations in these genes neither convincingly explains the disease in an individual who has been diagnosed with pancreatitis or has the ability to predict the possibility of developing the disease[88-90].

The significance of a positive test result for *PRSS1* genetic testing should be explained clearly to the subjects. Variable clinical course, mode of inheritance and incomplete penetrance are the important aspects apart from others, where counseling needs to be imparted to the patients. Strategies should be discussed to prevent future episodes of AP namely avoiding concomitant risk factors like alcohol, metabolic disturbances and drugs.

Important risk factors namely choledocolithiasis and other obstructive factors that contribute to AP have to be

identified and treated. Therefore patients have to be advised to undergo radiological and endoscopic evaluation to identify the above risks[91]. Furthermore, as these mutations (R122H or N29I) also significantly increase the risk for pancreatic cancer, the patients should be counseled for abstinence from tobacco and smoking[92] and counseling may be imparted and genetic testing ordered for at risk relatives if warranted[3].

CONCLUSION

As emphasized earlier many of the susceptibility loci identified till date have taken the candidate-gene approach and to the best of our knowledge there are no GWAS (Genome wide association studies) which are available apart from the only study which identified *PRSS1* and *CLDN2* polymorphisms recently[86]. Furthermore, a better understanding of the interactions of the etiological factors with susceptibility SNPs will aid in diagnosing and treating the disease at an early stage. There is an urgent need to utilize the advances in genomics namely GWAS and/or exome sequencing on NGS platform to unravel as yet unidentified susceptibility loci for pancreatitis, which is a multifactorial and a complex disease for a better understanding at the molecular level.

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