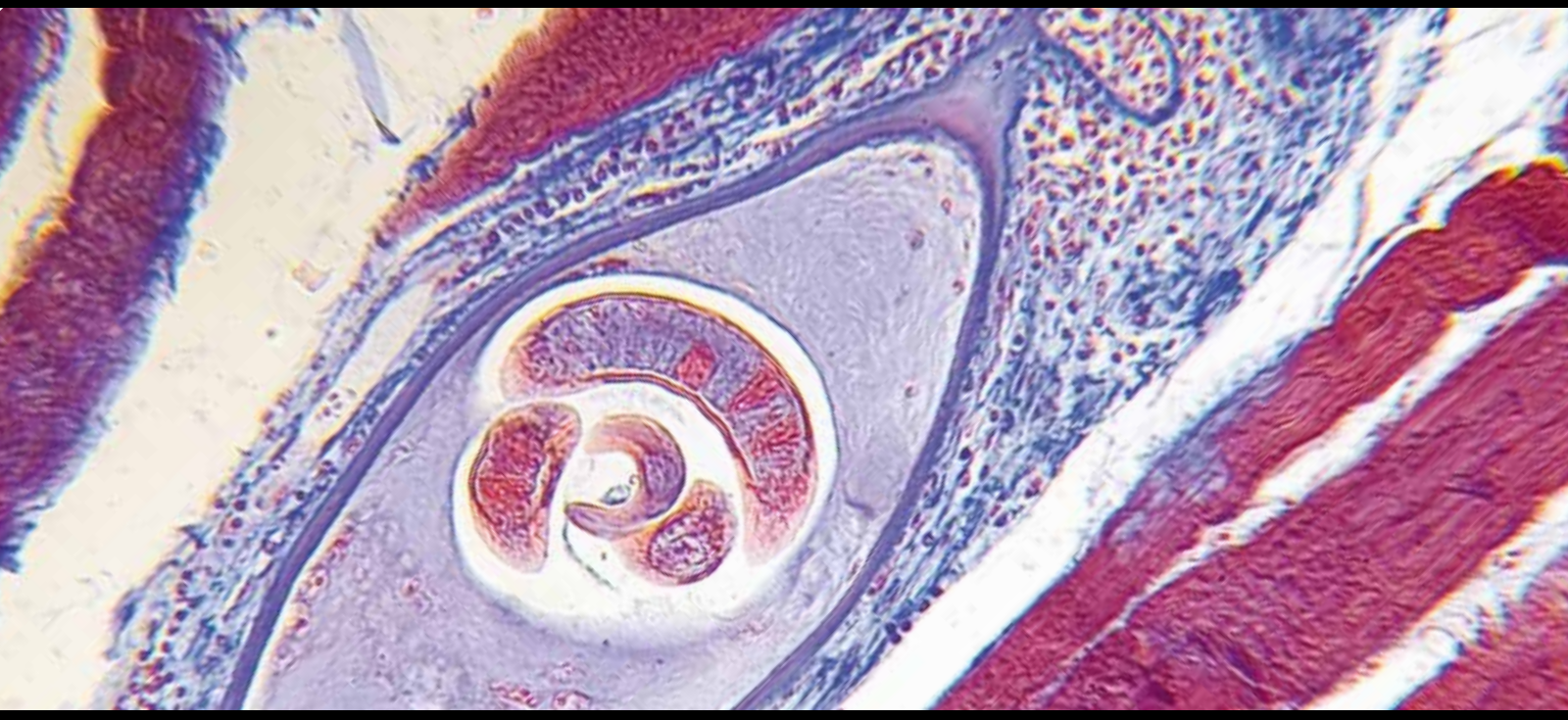
Gastroenterology Research and Practice

New Insights into Pathogenesis, Diagnosis, and Treatment of Pancreatic Disorders

Guest Editors: Grazyna Rydzewska, Jean Morisset, and Davor Stimac



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Editorial Board

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2013, Article ID 305179, 2 pages
http://dx.doi.org/10.1155/2013/305179

Editorial

New Insights into Pathogenesis, Diagnosis, and Treatment of Pancreatic Disorders

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Received 14 January 2013; Accepted 14 January 2013

Pancreatic disorders, such as acute and chronic pancreatitis and pancreatic cancer, are until now challenging diseases to manage, with significant burdens of morbidity, mortality rate, and high financial costs. Acute pancreatitis (AP) is still a common and potentially fatal disease with nonspecific treatment and unpredictable prognosis. In spite of significant improvement in understanding the basic pathophysiology of the disease and advances in the diagnosis and management of acute pancreatitis, the mortality rate has remained stable since the 1970s. Chronic pancreatitis (CP), resulting in a slow irreversible damage of the organ associated with gradual declines in digestive enzyme production, is the major cause of pancreatic exocrine insufficiency in adults. Finally, pancreatic cancer is known as a devastating process with only 6-month median survival after diagnosis.

In the present special issue,some recent advances in research concerning pancreatic diseases are presented. Croatian authors discussed epidemiology of AP in the North Adriatic Region of Croatia. They showed epidemiological characteristics typical of Mediterranean countries with predominant biliary etiology.

Early prediction of severity of acute pancreatitis (AP) by a simple unique laboratory test would be very helpful as it could direct management from the very beginning and at the end improve the outcome. Commonly accepted scales estimating the AP severity are extremely hard to use in clinical practice. For this reason, it is understandable why further attempts to find a parameter or a set of parameters useful in diagnosis and prognosis in AP are so necessary. Despite the importance of early prognosis, many patients initially identified as having a mild disease progress to severe acute pancreatitis (SAP) over time. Many biochemical parameters (including C-reactive protein (CRP), procalcitonin, and trypsinogen activation peptide (TAP)) have been evaluated in the severity assessment of AP.

In the present issue, authors discussed prognostic value of interleukins (IL) 6, 8, and 10, soluble receptor for tumor necrosis factor (sTNFr), pancreatic elastase (E1), and C-reactive protein (CRP) as predictors of systemic complications in patients with AP. They found that IL-6, IL-8, IL-10, and sTNFr measured on admission and CRP and pancreatic elastase measured on the third day of admission represent valuable prognostic factors in the determination of severity and systemic complications in patients with AP. Recently, parameters showing distortion of water balance in the course of AP have been a subject of research and clinical observations and were found very promising in severity and death prediction.

Although the pathogenesis of post-ERCP pancreatitis is not clearly understood, it seems that the patient's inflammatory response to pancreatic duct imaging and instrumentation plays a critical role. Several studies pointed out to

special factors, which put an individual in high risk for the development of post-ERCP pancreatitis. The history of post-ERCP pancreatitis as an independent risk factor for a new episode of post-ERCP pancreatitis seems to be very important. On the other hand, some individuals can have a genetically predisposed susceptibility for this particular complication. However, in the article published in this issue, the authors were not able to associate the role of SPINK1N34S mutation with post-ERCP pancreatitis.

Pancreatic cancer (PC) is one of the cancers of limited occurrence; however, worldwide over 200000 people die annually of this disease. The highest incidence and mortality rates of pancreatic cancer are found in developed countries. In the United States, pancreatic cancer is the fourth leading cause of cancer death, and in Europe it is the sixth. PC occurs three times as often in high-income countries as in those of middle and low income. Therefore, understanding the etiology and identifying the risk factors remain essential for the primary prevention of this disease. The risk factors, which can be easily eliminated, include tobacco smoking, obesity, and diabetes mellitus. The extent to which diet affects pancreatic cancer risk is still unclear.

The authors of the article included in the present issue have shown a correlation between PC and tobacco smoking (0.55 and 0.44). They also discussed that high consumptions of fats, sugar, and alcohol are potential risk factors for PC. Therefore, they suggest that positive changes in the diet such as lowering red meat consumption and increasing fruit consumption could influence incidence and reduction in future years.

Furthermore, other investigators discussed some interesting data concerning some microenvironmental elements, which could be involved in the development of pancreatic cancer. It is well known that growth of dense, collagen-rich, extracellular matrix and stroma, known as the desmoplastic reaction, creates a unique microenvironment promoting both tumour growth, and metastatic spread and forms a barrier to specific chemotherapy drug penetration. The authors discussed new developments in the fight against desmoplastic reaction, including inhibitors of the epidermal growth factor, fibroblast growth factor as well as new molecular targets like CD40 agonist and their effects on T cells, extracellular matrix modifying enzymes such as LOXL2 inhibitor, and novel tumor-penetrating peptides for delivery of drugs.

A group of scientists also described the involvement of macrophages in angiogenesis, process tumor growth and invasion of cancer cells. Macrophages are the source of angiogenic factors like VEGF and also MMP9, which degrade extracellular matrix. Therefore, macrophages infiltrating pancreatic tumor are another important factor promoting metastases. It was established that among many factors influencing tumor microenvironment c-Met receptor, infiltrating macrophages and MMP2 have significant influence on the development and invasion of pancreatic cancer.

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is now considered as one of the most useful methods for histological diagnosis and staging of pancreatic cancers. The Japanese discussed the usefulness, cost-effectiveness, and accuracy of EUS-FNA as a diagnostic modality for evaluating pancreatic tumors. They also underlined the role of molecular biological analyses for the diagnosis of PC. Other authors showed the utility of percutaneous ultrasonography-guided biopsy as a diagnostic tool for PC; however, it seems that this method should be rather reserved for nonresectable tumors.

We trust that the data presented in this special issue will help in understanding pancreatic disorder pathogenesis, especially microenvironment elements involved in the development of pancreatic cancer tumor. This hopefully will give new ideas for the development of new therapeutic strategies in pancreatic disorders.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2013, Article ID 956149, 5 pages
http://dx.doi.org/10.1155/2013/956149

Review Article

Epidemiology of Acute Pancreatitis in the North Adriatic Region of Croatia during the Last Ten Years

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Received 30 June 2012; Accepted 15 October 2012

Academic Editor: Grazyna Rydzewska

Introduction. Several European studies have reported an increase in the incidence rate of acute pancreatitis (AP). Therefore, we studied the incidence rate of AP in the North Adriatic Region in Croatia, as well as epidemiological analysis concerning etiology, age, gender, and severity of disease. *Methods*. We analyzed 922 patients with confirmed diagnosis of AP (history, clinical and laboratory findings, and imaging methods) admitted to our hospital during a ten-year period (2000–2009). Epidemiological analysis was carried out focusing on incidence, demographic data, and etiology, as well as severity of the disease based on the Ranson and APACHE II scores. *Results*. The incidence rate varied from 24 to 35/100 000 inhabitants annually. Mean age was 60 ± 16 years. There were 53% men and 47% women among the patients. Most frequent etiologies of AP were biliary stones in 60% and alcohol abuse in 19% of patients. According to the Ranson and APACHE II scores, pancreatitis was considered to be severe in 50% and 43% of the cases, respectively. *Conclusion*. In our region the incidence of AP was around 30 per 100,000 population per year during the ten-year period studied. The mean age at admission was 60 years and etiology was predominantly biliary. In our region, we have shown epidemiological characteristics of AP typical for Mediterranean countries.

1. Introduction

Acute pancreatitis (AP) represents an inflammatory disorder of the pancreas [1]. Due to the possibility of local and systemic complications, these patients are admitted to departments of internal medicine or surgical wards for further monitoring and treatment. The exact place of patient's admission depends on the country's tradition, or/and institutional work organization. Knowledge of the disease etiology is important, as early treatment can prevent local and systemic complications [2].

Incidence rate of AP varies in different parts of the world and the actual figures are mainly based on retrospective analyses of hospital admissions. Published studies have shown discrepant results in the incidence rates of AP, ranging from 10 to 80 new cases per 100,000 inhabitants annually [3–9]. There are considerable geographical differences, for example, a low incidence rate in the Netherlands [4] and UK [5] (10 and 24 pts/100,000 inhabitants/year), and a high incidence rate in the Scandinavian countries [6–8] and USA [9] (35 to 73 pts/100,000 inhabitants/year). There are also regional divergences with regard to the precipitating cause. In Finland [8] and USA [10] the main cause of AP is alcohol, whereas studies from Hong Kong [11], England [5], Italy, and Greece [12] showed biliary AP to be more common.

This is the first published study on the epidemiology of AP in the Croatian population. The aim was to determine the incidence of AP in the North Adriatic Region during a ten-year interval (2000–2009) and to analyze epidemiological factors (demographics, gender, age, and etiology) in patients with AP admitted to our hospital.

2. Patients and Methods

The incidence of AP in the North Adriatic Region of Croatia was calculated according to the 2001 census; there were 305,505 inhabitants (147,215 M/158,290 F) living in the region, and the incidence rate is presented as the number of new cases per 100.000 inhabitants.

The area of the region is 3.577 km^2 and the average population density is 85 inhabitants per square kilometer. The population is primarily urban, with the inhabitants living

in 14 cities. Being the referral center for pancreatic disease and the only hospital in our region, entire population of Northern Adriatic Region gravitate to our hospital. In our institution, patients with acute pancreatitis are hospitalized at the Department of Gastroenterology, Division of Internal Medicine.

All patients admitted to our hospital in the period from January 1, 2000, up to December 31, 2009, with a typical history including the onset of upper abdominal pain (nausea and/or vomiting) within 48 h prior to admission and the elevation of the serum amylase activity at least 3 times greater than the upper limit of normal, were considered to have AP.

Only the patients having the first attack of AP were included in the study. Patients with a relapse of AP or a relapse of chronic pancreatitis were excluded. The diagnosis of AP was additionally confirmed with imaging methods (abdominal ultrasound and/or CT scan), and in some patients hospitalized after year 2003, magnetic resonance (MR), magnetic resonance cholangiopancreatography (MRCP), or endoscopic ultrasound (EUS) were also done.

For the purpose of this epidemiological retrospective study patients were, according to etiology, divided into four groups: alcoholic, biliary, hypertriglyceridemic, and other. Biliary etiology was defined as the presence of gallstones determined by at least one of the imaging methods (abdominal ultrasound, CT, MRCP, or EUS). Alcoholic AP was considered in patients with confirmed alcohol consumption without cholelithiasis/choledocholithiasis, metabolic disorders (hypertriglyceridemia, hypercalcemia), or other possible causes of AP (trauma, drugs, etc.). Hypertriglyceridemia was considered as the cause of AP when the serum triglyceride level was above 11.3 μmol/L.

The severity of AP was determined by the APACHE II (on admission) and Ranson scores 48 hours upon admission [13]. Severe AP was considered if the APACHE II score was ≥8 and/or Ranson score was ≥3.

The collected data were formatted in a computer database using Microsoft Access (Microsoft Inc. USA), while statistical and data analysis was performed using statistical software MedCalc, 8th edition. We used χ^2-test for categorical data analysis and ANOVA for variance analysis. Multiple regression was used to determine independent predictors of severe acute pancreatitis. Incidence rate was calculated on 100.000 residents. P value < 0.05 was considered to be statistically significant.

3. Results

This epidemiological retrospective study included a total of 922 patients with AP.

There were 53% of men (mean age 59 ± 15) and 47% of a woman (mean age 63 ± 16). Although there is a similar occurrence of the disease between the two sexes in the ten-year period, we found that the first attack of the disease occurs at higher age in women than in men ($P < 0.001$). The incidence of acute pancreatitis in the North Adriatic Region for the period of 2000–2009 is presented in Figure 1.

The age distribution of the incidence of AP in the ten-year period in the North Adriatic Region is shown in Figure 2.

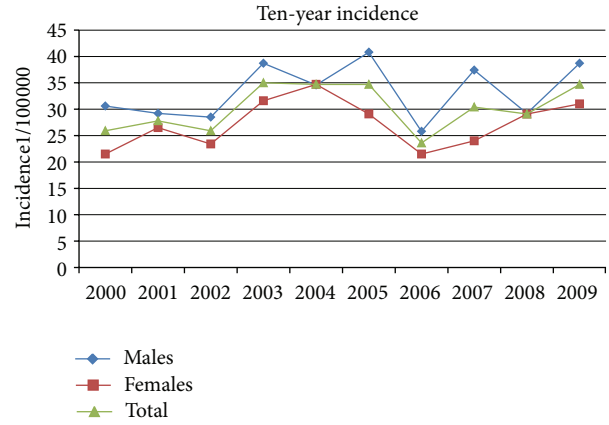

Figure 1: The incidence of acute pancreatitis according to gender during the ten-year period.

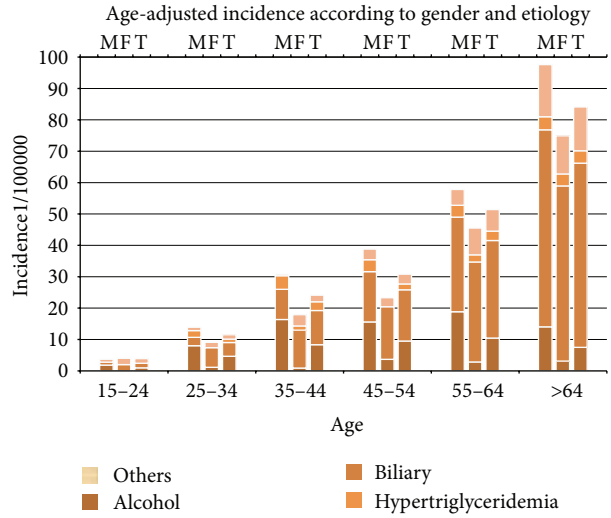

Figure 2: The incidence and etiology of acute pancreatitis change with aging. (M: male; F: female; T: total).

There is an obvious increase in the incidence of AP with age in both sexes. In men, this increase is more pronounced in the forties, while in women the incidence is higher in their fifties and sixties. In the analyzed group, biliary etiology was the most frequent cause of AP. Gallstones were the dominant cause of acute pancreatitis in both genders (Figures 2, 3(a), 3(b), and 3(c)). Although we did not find a significant difference in the occurrence of biliary pancreatitis between men and women, there were a significantly higher proportion of men having alcoholic pancreatitis (χ^2-test, $\chi^2 = 85.122$, $P < 0001$). There were no differences in the occurrence of certain etiologies within the specified time period. The ten-year period did not show any significant changes in the trend of respective etiologies (Figures 2 and 4.) The patients with the alcoholic etiology of the disease were in average younger than the other groups of patients ($P < 0.001$). The patients with the biliary etiology represent the oldest

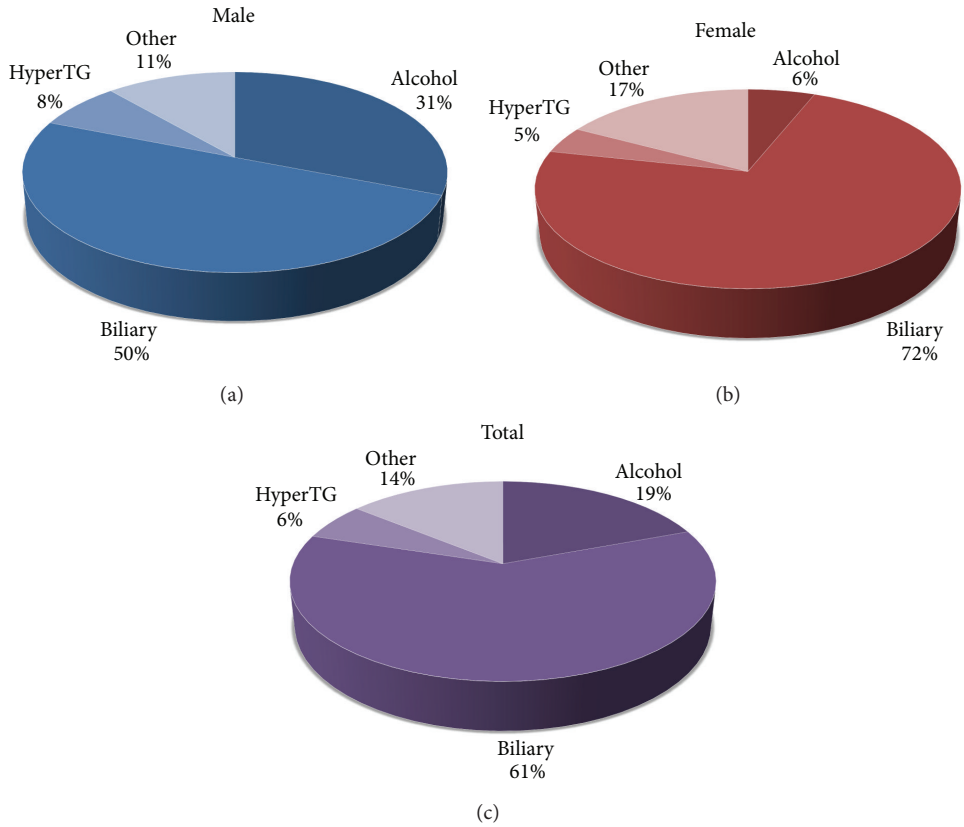

Figure 3: (a) The commonest causes of AP in men (HyperTG: hypertriglyceridemia). (b) The commonest causes of AP in female patients. One should note the rather low proportion of alcoholic AP as opposed to men (HyperTG: hypertriglyceridemia). (c) The commonest causes of AP in all patients (HyperTG: hypertriglyceridemia).

Table 1: The average age of patients with regards to the etiology of acute pancreatitis.

Etiology	Total		Male		Female	
	N (%)	Mean age ± SD	*N*	Mean age ± SD	*N*	Mean age ± SD
Alcohol	178 (19%)	52 ± 15	152 (31%)	50 ± 14	26 (6%)	59 ± 14
Billiary	558 (61%)	63 ± 15	246 (50%)	63 ± 14	312 (72%)	63 ± 16
Hypertriglyceridemia	56 (6%)	57 ± 16	37 (8%)	53 ± 15	19 (4%)	66 ± 15
Other	130 (14%)	61 ± 17	56 (11%)	63 ± 16	74 (18%)	60 ± 17

group of patients ($P < 0.001$) (Table 1). Thirteen patients had idiopathic pancreatitis.

The severity of pancreatitis was determined according to the Ranson and the APACHE II scores. According to the criterion of Ranson ≥3, 49% of patients had severe pancreatitis and 43% of patients, respectively, had severe AP with APACHE II score ≥8. There was no statistical significance among the various etiologies related to the severity determined by APACHE II and Ranson scores. Regression analysis showed that age was the only demographic factor that determined the severity of disease according to APACHE II and Ranson scores ($P < 0.001$).

4. Discussion

The incidence of AP ranges from 10 to 80 new cases/100,000 inhabitants wordwide, but in most European studies it has a much narrower range from 20 to 30 new cases/100,000 [3], that is, in accordance with the results of our study. Although several European studies have shown a significant increase in the incidence over the last 20 years, our study showed a steady trend over the entire ten-year period [2, 5, 14–18]. Our study has also shown an increase in the incidence of AP with regards to the age of the patients. The increase of the incidence with aging can be found in both sexes throughout the study period. The rate of increase differed between the sexes and was more pronounced after the age of 55 in women and after the age of 35 in men. Similar results were posted by the authors of the study conducted in Scotland (1961–1985), with the significant increase in the incidence in younger and middle-aged men (20–59 years) and in elderly women (60 years and older) [16]. In contrast to this findings, Floyd et al. [19] reported a much higher increase in women than in men between 1981 and

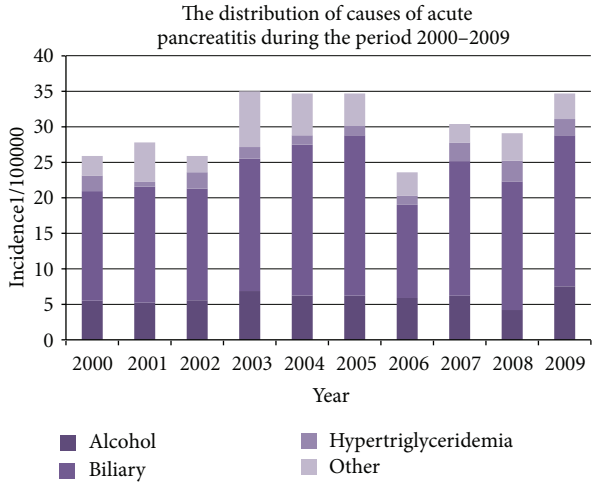

Figure 4: The distribution of the commonest etiologies of acute pancreatitis in the observed period.

2000 (women: 17.1–37.8; men: 18.0–27.1 per 100,000 inhabitants/year).

In the majority of the studies, gallstones were the predominant etiological factor of AP, except in Denmark (Copenhagen) [19] and Sweden (Goteberg [20] and Stockholm [21]) where alcohol was the predominate cause. Most of the studies have shown that specific etiologic factors dominate within a certain region of the country. For example, a higher proportion of cases with alcoholic AP were observed in the Grampian and Highland region of Scotland [22], compared with other regions in UK [23–26]. The different distribution of afore mentioned etiologies is not entirely clear but can be explained by the difference in alcohol consumption and incidence of cholelithiasis between North and South Europe. Continental Europe has a particularly high incidence of alcoholic pancreatitis, not only because of high alcohol consumption per capita, but also due to the fact that alcoholic beverages in this climate contain a high percentage of alcohol, and as such may have a stronger toxic effect on the pancreas. The frequency of alcoholic and biliary etiology varies throughout the study period, but without significant variances throughout the ten-year period, and as we would expect more women than man had biliary AP and more man than women suffered from alcohol-induced AP.

In our analysis there were 54 patients (6%) with hypertriglyceridemia as the cause of AP. According to the literature, it is assumed that hypertriglyceridemia is the cause of AP in 4% of cases [2, 27]. The mechanism of disease is not completely understood, but previous studies suggest that damage to pancreatic α cells or to the capillary endothelium could be caused by the action of free fatty acids [15]. These data are consistent with other epidemiological studies, especially with the results published in our neighboring country, Italy [12]. Living and eating habits in our county are similar to Italy and other Mediterranean countries and that explains the equal distribution of etiologies and other epidemiological characteristics.

The severity of pancreatitis was determined according to the Ranson score and the APACHE II score. Severe pancreatitis was considered in patients with Ranson score ≥3 and APACHE II score ≥8. Forty-nine percent of patients had severe pancreatitis according to Ranson score and fourty-three percent of patients according to APACHE II score. We found no statistical significance in disease severity graded by Ranson or APACHE II score, according to different etiologies of AP. Multivariate analysis showed age as the only demographic factor that determines the severity of disease according to both scores ($r = 0.2542$, $P < 0.001$), as expected. Although some studies have shown similar results, this phenomenon may be a consequence of a possible bias. Age is actually one of the necessary parameters for calculating the APACHE II and Ranson scores and thus makes a significant part in determining the severity of the disease in these two scoring systems. Also, the serum amylase values are highly dependent on the duration of symptoms prior to admission, and this gives high specificity but not a high sensitivity, so some cases might be missed, especially cases that are admitted late. Thus, some cases of mild pancreatitis are probably missed and this could explain, at least partialy, the high percentage of severe pancreatitis in our study.

5. Conclusion

In our region the incidence of AP was around 30 per 100,000 population per year during the ten-year period studied. Mean 10-year incidence was 30.2 (CI 95%; 24–36.4). Also, severity of disease was stable. In contrast to several European studies, the number of patients admitted to our hospital due to AP during the last ten years does not fluctuate significantly. Our study has shown that the North Adriatic Region has typical epidemiological characteristics of AP as neighbor Mediterranean countries like Italy and Greece.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2013, Article ID 282645, 6 pages
http://dx.doi.org/10.1155/2013/282645

Research Article

The Role of IL-6, 8, and 10, sTNFr, CRP, and Pancreatic Elastase in the Prediction of Systemic Complications in Patients with Acute Pancreatitis

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Received 30 June 2012; Accepted 26 November 2012

Academic Editor: Grazyna Rydzewska

Background and Aim. Early assessment of severity in acute pancreatitis (AP) is a key measure to provide rational and effective management. The aim of our study is to determine the prognostic value of interleukins (IL) 6, 8, and 10, soluble receptor for tumor necrosis factor (sTNFr), pancreatic elastase (E1), and C-reactive protein (CRP) as predictors of systemic complications in AP. *Patients and Methods*. A hundred and fifty patients with confirmed AP were enrolled in the study. The severity of AP was defined according to Atlanta criteria. Measurements of interleukins and sTNFr were performed on the first day of admission. CRP and E1 levels were assessed on admission and after 48 hours. ROC analysis was performed for all parameters. *Results*. Interleukins and sTNFr significantly differentiated patients with systemic complications from those without. Elevation of IL-6 showed the highest significance as a predictor ($P = 0.001$). CRP and elastase levels did not differ between mild and severe cases on admission, but reached statistical significance when measured on the third day ($P = 0.002$ and $P = 0.001$, resp.). *Conclusion*. Our study confirmed that IL-6, IL-8, IL-10, and sTNFr measured on admission, and CRP and pancreatic elastase measured on third day of admission represent valuable prognostic factors of severity and systemic complications of AP.

1. Background and Aim

Acute pancreatitis (AP) is a common and potentially lethal acute inflammatory disease with an estimated overall mortality rate of 2% to 5%, and a significant burden of morbidity and health care costs [1]. Although usually self-limiting, up to 20% of patients develop a severe form of disease, which can lead to a systemic inflammatory response and multiple organ dysfunction and failure [2]. The two prevailing causes of AP are excessive alcohol consumption, most common in men, and gallstones, most common in women, which seem to act through different pathogenic mechanisms to induce pancreatic acinar cell damage. Several multifactorial scoring systems and routine clinical and biochemical parameters measured on admission and during the first 48 hours of hospitalization are used to estimate severity and promptly provide a rational and effective management.

Systemic manifestations of a disease initially limited to the pancreas are thought to be mediated by a variety of pro- and anti-inflammatory mediators released from the pancreas and various other sources during the course of the disease. Several cytokines play a crucial role in the pathogenesis of AP by driving the additional inflammatory response which leads to tissue damage and organ dysfunction. Local recruitment and activation of inflammatory cells in AP may lead to the production of proinflammatory cytokines, such as interleukins (IL) 6, 8, and tumor necrosis factor alpha (TNF-alpha) or his soluble receptor (sTNFr), as well as anti-inflammatory IL-10. These mediators have been mostly studied as markers of severity of acute pancreatitis. Another commonly applied and one of the first used markers for this purpose was C-reactive protein (CRP). Different studies showed that a CRP value over 200 mg/L obtained at 48 hours after onset of symptoms is highly predictive of pancreatic necrosis [3].

The use of pancreatic elastase for the differentiation between mild and severe cases of AP has also been investigated, yielding however conflicting results [4].

Still, insufficiently is known of the relationship between the clinical course of AP in humans and the dynamic of the major cytokines, in the presence or absence of pancreatic necrosis and distant organ complications. The purpose of our study was to determine the potential clinical value of interleukins (IL-6, IL-8, IL-10), soluble receptor for tumor necrosis factor (sTNFr), pancreatic elastase, and C-reactive protein as biochemical markers for predicting development of systemic complications in patients with AP.

2. Patients and Methods

2.1. Patients. A hundred and fifty patients with acute pancreatitis were prospectively entered into the study during a two-year period. The diagnosis of AP was made on the basis of a consistent clinical picture combined with a 3-fold increase of serum amylase or a 3-fold increase of serum lipase, and consistent morphological findings obtained by an ultrasound scan and/or computed tomography scan within the first 72 hours of admission. The severity of AP was assessed according to the Atlanta classification. All patients irrespective of disease severity were included in the study. Mild acute pancreatitis (MAP) was defined as confirmed AP without signs of major complications, while severe acute pancreatitis (SAP) was associated with the development of one or more local or systemic complications. Local complications included pancreatic tissue necrosis, as well as the formation of acute fluid collections, pancreatic pseudocyst, and abscess. Systemic complications assumed the presence of persistent systemic inflammatory response syndrome (SIRS) and/or developing organ failure. SIRS was defined by 2 or more of the following criteria for >48 hours: heart rate >90 beats/min; rectal temperature <36°C or >38°C; white blood count <4000 or >12,000 per mm^3; and respirations >20/min or pCO_2 < 32 mmHg. Organ failure was defined as shock (systolic blood pressure <90 mmHg), pulmonary insufficiency (pO_2 < 60 mmHg), renal failure (creatinine >2 mg/dL, despite rehydration), and gastrointestinal bleeding (>500 mL/24 hours) [5, 6]. The study was performed according to local ethics committee regulations.

2.2. Methods. Blood samples from patients were obtained on admission and after 48 hours. Samples for IL-6, IL-8, IL-10, IL-15, IL-17, and sTNFr were aliquoted in portions and stored at −20°C, not longer than two months. Measurements were performed using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, USA) on a standard ELISA reader according to the manufacturer's instructions. Pancreatic elastase was analysed using a commercially available ELISA kit (ScheBo-Biotech, Giessen, Germany). Levels of CRP and other routine laboratory assessments were completed on biochemistry analyser Olympus AU 640 (Mishima Olympus, Japan). Measurements of interleukins and sTNFr were performed on samples obtained on the first day of admission. CRP and pancreatic elastase levels were assessed on admission and after 48 hours.

2.3. Statistics. All variables are expressed as medians with 95% cofidence intervals (95% CI). Mann Whitney *U* test was used for comparison of independent samples. For differences between values of same parameters obtained on admission and after 48 hours Wilcoxon pair test for dependent samples was used. Receiver operating characteristic (ROC) curves and respective areas under curve (AUC) were established for biochemical prognostic factors. Cut-off values were chosen as values that achieved the highest sensitivity and specificity, as well as positive (PPV) and negative predictive values (NPVs). The proportion of patients without systemic complications was used as a measure of prevalence in performing ROC analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

A total of 150 patients with AP (71 male; median age 63; range 20–91) were included in the study. AP was considered severe in 28 patients (19%) and mild in 122 patients (81%). The etiology of AP was biliary in 68 patients (45%), alcoholic in 51 patients (34%), and other possible causes (hypertriglyceridemia, post-ERCP, idiopathic, etc.) in 31 patients (21%).

The average value of IL-6 measured in the group of patients was higher than the upper limit of reference range recommended by the manufacturer (29 versus 12.5 pg/mL, resp.), whereas average values of other measured cytokines were within normal ranges. CRP measured on the first and third day of admission was above the upper limit of normal, as well as the average value of pancreatic elastase measured on the first day. Average values of pancreatic elastase measured on the third day were within the boundaries of recommended values (Table 1).

In the assessment of disease severity, average values of CRP and pancreatic elastase differed significantly between the first and third day of hospitalization, with a significant increase in CRP values and a significant decrease in serum concentrations of pancreatic elastase (Table 2).

The comparison of the analyzed biochemical prognostic factors between acute pancreatitis patients who developed systemic complications and those who did not is shown in Table 3. We found a significant difference between the values of IL-6, IL-8, IL-10, and sTNFr evaluated on the first day of admission, and a significant difference between CRP and elastase values analyzed from samples taken on the third day. No significant difference was noted in the values of CRP and elastase on the first day between these two groups of patients.

The effectiveness of the investigated biochemical parameters in the early recognition of patients with and without systemic complications was assessed using ROC analysis. Considering the area under the ROC curve, values of cytokines measured on the first day were statistically significant indicators for development of systemic complications. CRP and pancreatic elastase measured on the third day

Table 1: Average values of biochemical parameters in patients with acute pancreatitis and the respective reference values according to manufacturers' recommendations.

Parameters	Median (95% CI)	Reference values
IL-6 (1st day)	**29 (18–46)**	**3.13–12.5 pg/mL**
IL-8 (1st day)	30 (23–39)	≤31.2 pg/mL
IL-10 (1st day)	7.4 (3.9–11.2)	≤7.8 pg/mL
sTNFr (1st day)	1698 (1419–1998)	749–1966 pg/mL
CRP (1st day)	**15.2 (12–27)**	**≤5 mg/L**
CRP (3rd day)	**103.6 (80–139)**	**≤5 mg/L**
Elastase (1st day)	**5.9 (4.3–7.5)**	**≤3.5 ng/mL**
Elastase (3rd day)	1.8 (1.6–2.2)	≤3.5 ng/mL

Table 2: Significant difference of average CRP and elastase values measured on the first and third day of admission in patients with acute pancreatitis.

Parameters	Median (95% CI)	P value
CRP (1st day) [mg/L]	15 (12–27)	$P < 0.001$
CRP (3rd day) [mg/L]	104 (80–139)	
Elastase (1st day) [ng/mL]	5.9 (4.3–7.5)	$P < 0.001$
Elastase (3rd day) [ng/mL]	1.8 (1.6–2.2)	

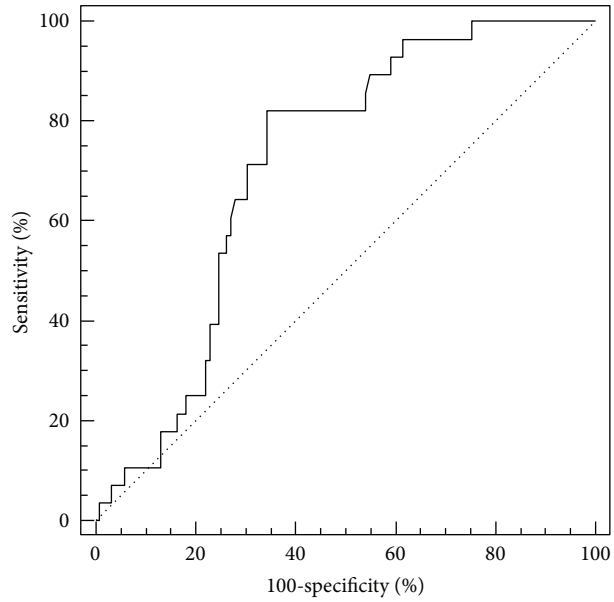

Figure 1: ROC curve for IL-6. Area under the curve (AUC) = 0.71; the limit value is 37.9 pg/mL.

also reached statistical significance. Results from analysis are shown in Table 4.

The largest area under the curve was for IL-6 (AUC = 0.71) and elastase on the third day (AUC = 0.70) (Figures 1 and 2). Elastase had a fairly high sensitivity of 92%, but a rather low specificity of 43%. The highest specificity (84%) was calculated for the marginal value of CRP measured on the third day, with a sensitivity of 54% (Figure 3). CRP and elastase measured on the first day had the lowest predictive value (AUC = 0.51 and 0.56, resp.) not reaching statistical significance.

4. Discussion

In this study we examined the value of IL-6, IL-8, IL-10, sTNFr, CRP, and pancreatic elastase as predictors of systemic complications in AP. The need for an early risk recognition and determination of best possible treatment modalities led to a series of investigations trying to establish an objective, rational, and clinically manageable severity assessment tool in patients with AP.

The initial acinar cell damage in the early stage of acute pancreatitis of any etiology is caused by a hypersecretion of pancreatic proteolytic enzymes. As a result there is an overproduction of inflammatory mediators and free oxygen radicals. Tissue macrophages are the main source of proinflammatory and anti-inflammatory cytokines that attract neutrophils and more macrophages, and induce the production of proteases, elastases, and phospholipases. These enzymes, as well as free oxygen radicals cause tissue damage, mainly vascular endothelial necrosis which leads to circulatory stasis. The increase of proinflammatory and decrease of anti-inflammatory cytokines are crucial factors in the progression of inflammation of severe acute pancreatitis. The largest studies have focused on the role of TNF-alpha, IL-1, IL-6, and IL-10. Most of these studies have shown that the levels of proinflammatory cytokines (TNF-alpha, IL-1, IL-6) are higher in severe forms of AP, while levels of IL-10, which is anti-inflammatory agent are higher in patients with mild disease. The systemic manifestations of severe acute pancreatitis are not only caused by local inflammatory processes, but also by an excessive production and systemic spreading of inflammatory mediators.

We performed data analysis by dividing our patients into two groups. In one group we had patients who developed systemic complications ($N = 28$), and in the other those who had none ($N = 122$). The proportion of patients with systemic complications in our study correlates with the published data [7]. Our results show that the average value of IL-6 in patients with AP was above the upper limit of reference range recommended by the manufacturer, while average levels in controls were within normal ranges. ROC analysis was performed to evaluate the prognostic value of IL-6 to distinguish patients with systemic complications from those without, showing that patients with IL-6 concentrations greater than 37.9 pg/mL can be considered high risk in terms of developing systemic complications. We found a sensitivity of 82%, and a specificity of 65%, with a PPV of 35%, and an NPV of 94%. In our previous study, results differed slightly with a sensitivity and specificity of 68.7% and 69.9%, respectively, and PPV of 50%, and NPV of 83.6% [8]. These differences probably derive from the quality of available tests. Pezzilli et al. showed in their paper an AUC of 0.91, a sensitivity of 100%, and specificity of 83% [9]. These values in combination with serum lipase levels achieved a diagnostic and prognostic accuracy of 94%. The most likely cause is a smaller number of patients and the

Table 3: Values of biochemical parameters measured in patients without systemic complications ($N = 122$) and patients who developed systemic complications ($N = 28$) and comparison of measured values. Bold printed are the indicators with statistically significant difference.

Parameters	Patients without systemic complications ($N = 122$) Median (95% CI)	Patients with systemic complications ($N = 28$) Median (95% CI)	P value
IL-6 (1st day) [pg/mL]	20 (16–34)	104 (51–139)	**0.001**
IL-8 (1st day) [pg/mL]	27 (19–34)	53 (33–98)	**0.012**
IL-10 (1st day) [pg/mL]	5.1 (2.6–8.9)	26 (8–58)	**0.010**
sTNFr (1st day) [pg/mL]	1520 (1220–1886)	2220 (1873–2722)	**0.004**
CRP (1st day) [mg/L]	14.4 (11.3–28.3)	15 (5.1–78.8)	0.954
CRP (3rd day) [mg/L]	85 (66.7–122)	216 (105–257)	**0.002**
Elastase (1st day) [ng/mL]	5.7 (4.2–7.5)	7.5 (3.6–10.9)	0.249
Elastase (3rd day) [ng/mL]	1.7 (1.4–1.9)	2.5 (2.1–5.1)	**0.001**

Table 4: ROC analysis for biochemical parameters of patients with acute pancreatitis. The criterion of classifying patients is the occurrence or absence of systemic complications of the disease. Bold printed are the indicators with statistically significant difference.

Parameters	AUC (95% CI)	P value	Cut-off	Sensitivity (%)	Specificit (%)	PPV (%)	NPV (%)
IL-6 (1st day) [pg/mL]	0.71 (0.63–0.78)	<**0.001**	37.9	82	65	35	94
IL-8 (1st day) [pg/mL]	0.66 (0.58–0.74)	**0.008**	42.5	68	67	32	90
IL-10 (1st day) [pg/mL]	0.65 (0.57–0.73)	**0.013**	7.2	75	56	28	91
sTNFr (1st day) [pg/mL]	0.69 (0.60–0.76)	**0.002**	1552	82	53	29	93
CRP (1st day)	0.51 (0.43–0.60)	0.842	85	32	86	35	85
CRP (3rd day) (mg/L)	0.69 (0.61–0.77)	**0.001**	214	54	84	44	89
Elastase (1st day) (ng/mL)	0.56 (0.48–0.64)	0.311	2.0	92	22	22	93
Elastase (3rd day) (ng/mL)	0.70 (0.62–0.77)	<**0.001**	1.5	93	43	27	96

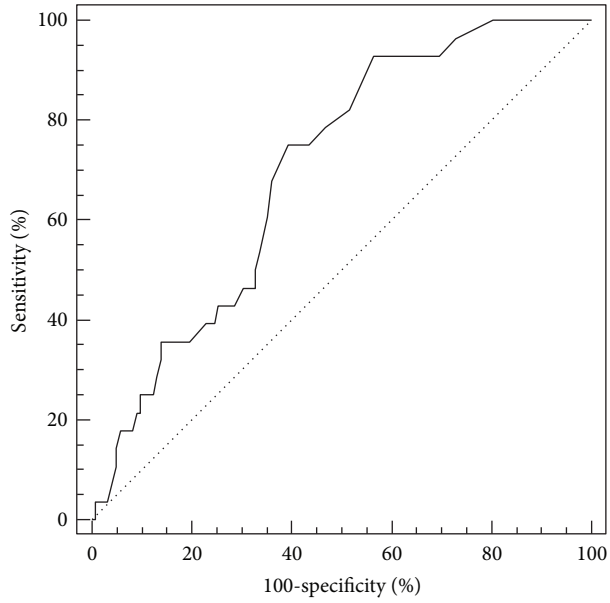

Figure 2: ROC curve for elastase measured on the third day of admission. Area under the curve (AUC) = 0.70; the limit value is 1.5 ng/mL.

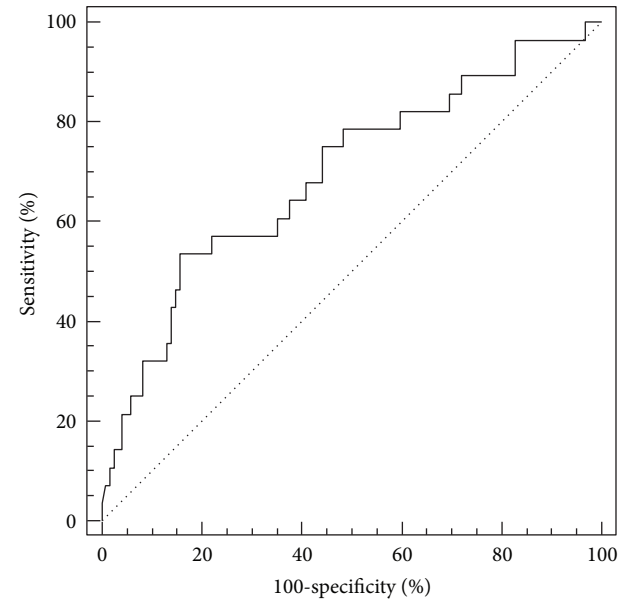

Figure 3: ROC curve for CRP measured on the third day of admission. Area under the curve (AUC) = 0.69; the limit value is 214 mg/L.

use of different tests for cytokine analysis. In another study, Jiang et al. determined the concentration of IL-6, TNF-alpha, and CRP over several days after admission and found that IL-6 has the highest sensitivity and specificity (100% and 89.7%, resp.) on the first day of admission [10]. Chen et al. analyzed, among other things, levels of IL-6, IL-8, and IL-10 in 78 patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) and found that patients

who suffered from post-ERCP pancreatitis have significantly higher concentrations of these cytokines [11]. Using a cut-off level of 36 pg/mL they found that the sensitivity and specificity for recognition of post-ERCP pancreatitis were 100% and 87%, respectively.

The role of the proinflammatory IL-8 in prediction of severity of acute pancreatitis seems less valuable than IL-6. Although it reached statistical significance ($P < 0.008$) in the differentiation of mild and severe disease forms at a threshold value of 42.5 pg/mL, it achieved a modest sensitivity and specificity of 68% and 67%, respectively. Same conclusions were obtained in previous studies by Pooran et al., and Berney et al. [12, 13].

Interleukin 10 has an anti-inflammatory role inhibiting the synthesis and release of other proinflammatory cytokines and free oxygen radicals from macrophages and T-helper lymphocytes. On the other hand, it shows a positive effect on the proliferation and differentiation of B lymphocytes promoting the production of immunoglobulins. Our results show that a limit for IL-10 that can be said to separate milder from more severe forms of AP is 7.2 pg/mL, with a sensitivity of 75%, and a specificity of 56%. Our previous study showed a lower sensitivity, and same specificity for a higher cut-off value (37 pg/mL) [8]. These differences are most probably related to the already mentioned different sensitivity of various commercial tests. A statistically significant elevation of IL-10 in patients with severe AP was obtained by other authors as well [14–16]. However, two other studies found significantly lower values of IL-10 in patients with severe AP [17, 18]. Authors speculated that an impaired immune response to inflammation could be a possible cause. It seems that a balance between pro- and anti-inflammatory cytokines is the key process in the course of AP and development of systemic complications. A reduced functional reserve of IL-10 and a higher IL-6/IL-10 ratio could lead to SAP and a worse prognosis. However, this is still a matter requiring further investigations.

Results of sTNFr analysis, as another proinflammatory cytokine, were consistent with previously published results showing a significant elevation of serum concentrations in patients with SAP [19].

The research included the analysis of two parameters that can be considered as valuable indicators of the course of disease; pancreatic elastase as a specific enzyme secreted by pancreatic acinar cells, and CRP as an acute phase protein, both increasingly produced and released in a state of acute inflammation. Elevated concentrations of E1 were measured in both groups of patients on admission, but without significant difference between the groups. However, we noticed a significant decline in concentration of E1 between the first and third day ($P < 0.001$), with a significant difference in values between the two groups patients ($P = 0.001$). Our results show that patients with a value of E1 below a cut-off value of 1.5 ng/mL measured on third day of admission could be considered potentially at low risk of development of systemic complications.

A group of Australian authors in their examination of E1 concentrations in 29 patients with acute pancreatitis showed a sensitivity of 80%, and a sensitivity of 96% for a E1 cut-off value of 3.5 ng/mL measured on admission, with a sensitivity and specificity of 100% and 96%, respectively, on day three. The authors stated concurring limitations of ELISA tests, including lack of sensitivity due to questionable quality of used antibodies, and problematic reference range given by the manufacturer [20]. Similar limitations are present in all studies measuring serum concentrations of small amounts of antigens. Therefore, ELISA methods are not standardized and recommended for routine cytokine and elastase analyses, and should be used only for research purposes. Moreover, the implementation of the analysis in larger series, which includes sample collection and subsequent determination, makes these methods unsuitable for routine use. Development of homogenous immunochemical methods appropriate for automated and standardized determination of individual samples should increase the prognostic value and practical application of different biochemical parameters.

As well as for E1 analysis, the concentrations of CRP differed significantly between day one and three ($P < 0.001$). Patients who developed systemic complications showed significantly higher levels of CRP ($P = 0.002$). ROC analysis showed and AUC of 0.69 for a cut-off value of 214 mg/L, with a rather low sensitivity and specificity, 54% and 84%, respectively. Gürleyik et al. showed similar results with sensitivity of 85%, and specificity of 74%, although for a cut-off value that was significantly different than in our study, 193 mg/L versus 214 mg/L, respectively [21]. Other authors also point out the value of CRP as a tool for AP severity assessment [22–24].

We confirmed that CRP and elastase analyzed on the third day of admission, in addition to the evaluation of IL-6, IL-8, IL-10, and sTNFr on the first day, represent a valuable diagnostic tool in the assessment of severity and course of disease in patients with acute pancreatitis. Nevertheless, CRP is still the only recommended and standardized method for a fast and relatively inexpensive determination of severity of AP. Routine use of proinflammatory cytokines as predicting factors of severity of acute pancreatitis is still not feasible in most hospitals, due to high costs and inaccessibility of analytic methods. Therefore, development of new and more accessible laboratory equipment, as well as methods of analysis could help the clinicians in the early recognition of development of systemic complications and improve the management of severe acute pancreatitis.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 682156, 9 pages
doi:10.1155/2012/682156

Research Article

Influence of Diet and Tobacco Smoking on Pancreatic Cancer Incidence in Poland in 1960–2008

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Received 25 May 2012; Revised 12 November 2012; Accepted 22 November 2012

Academic Editor: Grazyna Rydzewska

The aim of the study was to investigate the relationship between pancreatic cancer incidence and selected dietary factors, alcohol consumption, and tobacco smoking in Poland in 1960–2008. Data on pancreatic cancer morbidity were derived from the National Cancer Registry and on food consumption from the national food balance sheets. In 1960–1989 correlations were found between pancreatic cancer incidence rates and energy (0.60 for males and 0.57 for females), cholesterol (0.87 and 0.80), fibre (−0.84 and −0.89) and folate (−0.45 and −0.49) intake, the consumption of total fats (0.94 and 0.91), animal fats (0,90 and 0,82), sugar (0.88 and 0.87), cereals (−0.93 and −0.91), and alcohol (0.86 and 0.82). In 1990–2008 morbidity correlated with the consumption of red meat (0.67 and 0.48), poultry (−0.88 and −0.57), and fruit (−0.62 and −0.50). Correlation with tobacco smoking was observed in the whole studied period (0.55 and 0.44). Increased incidence of pancreatic cancer in 1960–1995 was probably related to adverse dietary patterns up to 1989, especially high consumption of fats, sugar, and alcohol. Further positive changes in the diet such as lowering red meat consumption and increasing fruit consumption could influence incidence reduction in recent years. Also changes in tobacco smoking could affect the morbidity.

1. Introduction

Pancreatic cancer is one of the cancers of limited occurrence. It mostly concerns high-income countries, where it occurs three times as often as in middle- and low-income countries [1].

In its early stage symptoms are barely observable, and, when diagnosed, the disease is usually in an advanced stage [1, 2]. Therefore survival rates are low; 90% of patients die within 12 months from the moment of diagnosis [3]. In Europe pancreatic cancer is the 6th leading cause of cancer death [4]. In Poland it is ranked 7th among men and 6th among women in the list of cancers that are most frequent causes of death [5].

In that situation understanding the etiology and identifying the risk factors is essential for the primary prevention of this disease. Potentially modifiable risk factors include tobacco smoking, obesity, and diabetes mellitus [3, 6]. The extent to which diet affects pancreatic cancer risk is still unclear. Among nutritional factors that can reduce or increase the risk of pancreatic cancer, the following are listed most often: consumption of food containing folate, fruit, red meat, cereals, and fat [1]. Another important factor is probably chronic pancreatitis, the main cause of which is alcohol consumption [7, 8].

Prevention could play an important role in reducing pancreatic cancer mortality, and an important question is whether diet interventions can lower the risk. Only long-term studies covering several dozens of years, examining how dietary pattern changes affect incidence rates, can ensure a chance to make an objective assessment of this matter.

The present paper is an attempt to investigate the relationship between the changes in pancreatic cancer incidence rates and selected dietary factors, alcohol consumption, and tobacco smoking in the period from 1960 to 2008.

2. Methods

Data on pancreatic cancer incidence rates were derived from the National Cancer Registry administrated by the Maria

Table 1: Correlations between dietary factors and pancreatic cancer morbidity rates, 1960–1989, 1990–2008, 1960–2008, by sex.

Factor	1960–1989		1990–2008		1960–2008	
	Men	Women	Men	Women	Men	Women
	r (P value)	r (P value)	r (P value)	r (P value)	r (P value)	r (P value)
Energy	0.60 (<0.001)	0.57 (<0.01)	−0.29 (0.263)	−0.47 (0.055)	−0.50 (<0.001)	−0.57 (<0.001)
Total edible fat	0.94 (<0.001)	0.91 (0.001)	−0.87 (<0.001)	−0.63 (0.007)	0.75 (<0.001)	0.79 (<0.001)
Animal fats	0.90 (<0.001)	0.82 (<0.001)	0.60 (0.011)	0.30 (0.267)	−0.08 (0.616)	−0.24 (0.115)
Cholesterol	0.87 (<0.001)	0.80 (<0.001)	−0.52 (0.033)	−0.62 (0.008)	0.32 (0.035)	0.24 (0.107)
Sugar	0.88 (<0.001)	0.87 (<0.001)	0.24 (0.347)	−0.01 (0.981)	0.49 (<0.001)	0.40 (0.007)
Red meat	0.34 (0.342)	0.19 (0.193)	0.67 (0.003)	0.48 (0.050)	0.14 (0.445)	−0.03 (868)
Poultry	0.40 (0.180)	0.34 (0.248)	−0.88 (<0.001)	−0.57 (0.016)	0.15 (0.437)	0.39 (0.033)
Fruit	0.50 (0.007)	0.36 (0.059)	−0.62 (0.008)	−0.50 (0.039)	0.59 (<0.001)	0.64 (<0.001)
Cereals	−0.93 (<0.001)	−0.91 (<0.001)	0.52 (0.033)	0.56 (0.021)	−0.82 (<0.001)	−0.81 (<0.001)
Dietary fibre	−0.84 (<0.001)	−0.89 (<0.001)	0.44 (0.079)	0.38 (0.136)	−0.84 (<0.001)	−0.87 (<0.001)
Folate	−0.45 (0.017)	−0.49 (0.009)	0.71 (0001)	0.36 (0.151)	−0.58 (<0.001)	−0.68 (<0.001)
Alcohol	0.86 (<0.001)	0.82 (<0.001)	−0.87 (<0.001)	−0.57 (0.017)	0.48 (<0.001)	0.51 (<0.001)
Tobacco	0.87 (<0.001)	0.82 (<0.001)	0.72 (0.001)	0.41 (0.100)	0.55 (<0.001)	0.44 (0.003)

Skłodowska-Curie Memorial Cancer Center and Institute of Oncology in Warsaw [5, 9]. They showed age-standardized incidence rates for men and women covering years between 1960 and 2008 excepting 1984, 1986, 1997, and 1998, for which no such data were available. "Standard global population" was taken as a standard.

The source of information on the dietary pattern in the same period was the database established by the National Food and Nutritional Institute [10–12]. This database covers data derived from the national food balance sheets and shows major food groups quantities available for consumption per capita/year. The population averages for consumption do not give separate values for men and women, nor actual consumption at the individual level. The methodology of the food balance sheets preparation is fairly harmonized internationally due to the efforts of the FAO [13]. The data on food group consumption are converted into energy and nutrients intake (per person/day) with the use of the national food composition tables [14].

Information on alcohol consumption (as total expressed in pure alcohol and including consumption of major types of alcoholic beverages and their alcohol content) and tobacco smoking showing number of cigarettes per capita/year was derived from national statistical year books [15].

The study was focused on identification and measurements of the relationship between pancreatic cancer incidence rates and variables related to dietary pattern represented by the intake of energy, folate, cholesterol and dietary fibre and the consumption of red meat, poultry, fruit, total and animal fats, cereals, and sugar. Data on red meat consumption covering individual years were available from 1974 onwards while earlier information in this regard included 1960 and 1970 only. There was also lack of data on poultry consumption in 1961–69, 1971–74, and 1976-77. Moreover possible influence of alcohol consumption and cigarette smoking on pancreatic cancer morbidity was analyzed.

The relationship between the above risk factors and pancreatic cancer incidence rates was analyzed for the whole period of 1960–2008 and for two subperiods: 1960–1989 and 1990–2008, due to the courses and dynamics of dietary pattern changes before and after the economic transformation.

Spearman's rank correlation coefficients (r) were estimated as a measure of the relationship between pancreatic cancer incidence rates and selected parameters.

3. Results

Pancreatic cancer is more frequent among men than among women. In 2008 in Poland a standardized incidence rate amounted to 6.1/100 thousand for males and 3.9/100 thousand for females [5]. Between 1960 and 1995 pancreatic cancer incidence among men increased from 1.8/100 thousand to 8.6/100 thousand and from 1.1/100 thousand to 5.0/100 thousand among women [5, 9]. Since then, a favourable downward trend has been observed, which is more evident among men.

Correlation coefficient (r) and P values between dietary factors or tobacco smoking and pancreatic cancer incidence rates are presented in Table 1.

One of the factors increasing the risk of pancreatic cancer is excessive adipose tissue, accrued, among others, as a result of a diet with excessive energy content. Up to 1989 the energy content of a typical Polish diet was relatively high (Figure 1) and correlated positively with the increasing incidence of pancreatic cancer (correlation coefficient of 0.60 among males and 0.57 among females). Reduced energy intake on the turn of the 80s and 90s of the 20th century may have positively influenced the reduced incidence although no correlation between both variables had been observed.

Consumption of fats, including animal fats, may have been related to pancreatic cancer incidence. Since 1960s the increased overall consumption of fats took place (Figure 2).

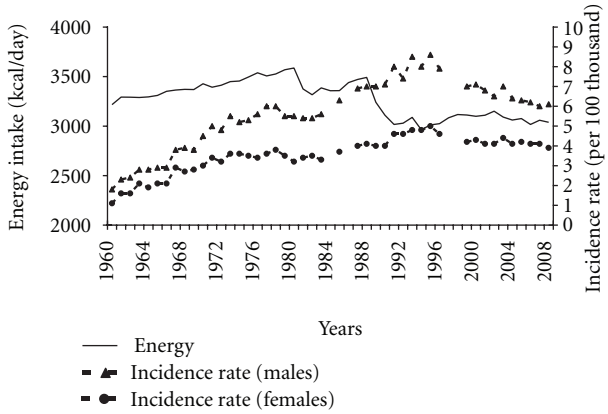

Figure 1: Energy intake and pancreatic cancer morbidity 1960–2008.

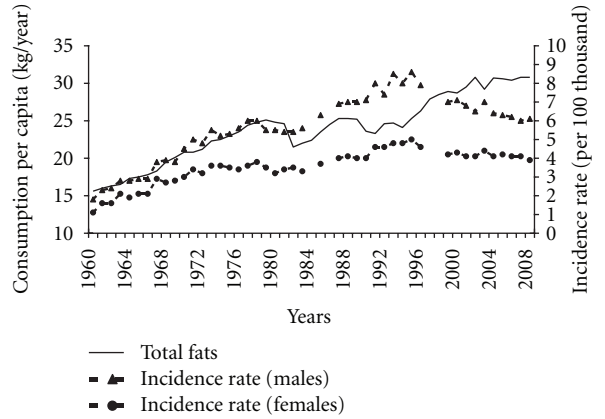

Figure 2: Total fats consumption and pancreatic cancer morbidity 1960–2008.

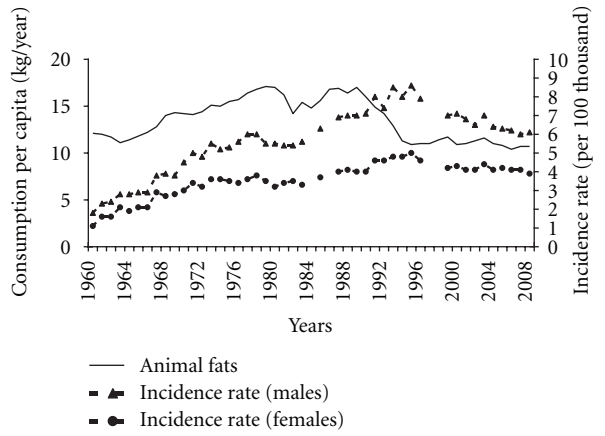

Figure 3: Animal fats consumption and pancreatic cancer morbidity 1960–2008.

Between 1960 and 1989 the correlation between the consumption of these products and the incidence was almost complete (0.94 among males and 0.91 among females). Incidence also correlated positively with the escalating consumption of animal fats (0.90 among males and 0.82 among females). After the transformation the structure of fats consumption changed and the share of animal fats in a diet significantly fell (Figure 3).

In the period after the transformation changes concerning the cholesterol content in a diet also took place (Figure 4). Between 1960 and 1989 the cholesterol intake correlated positively with incidence rates (0.87 among males and 0.80 among females). Reduced cholesterol intake in later years may have been one of the factors contributing to the reduced incidence of pancreatic cancer, despite the lack of positive correlation between these variables.

Before 1995 one of the unfavourable factors contributing to the increased pancreatic cancer incidence had probably been the sugar consumption, which had initially been growing to remain at a high level (Figure 5). In the years 1960–1989 the sugar consumption correlated positively with incidence rates (0.88 among males and 0.87 among females). Although in later years no correlation between these variables was found, we cannot rule out that the reduced sugar consumption may have beneficially influenced the incidence trends.

The consumption of red meat is a factor increasing pancreatic cancer risk. After 1960 the red meat consumption was significantly growing and although later on some fluctuations were observable it remained high (Figure 6). In spite of the lack of correlation, it cannot be ruled out that its high consumption had a certain impact on the morbidity in the years 1960–1989. Between 1991 and 1997 the red meat consumption decreased and stabilized on a lower level than before. This might have been one of the reasons for the reduction in incidence after 1995, which is demonstrated by a positive correlation between these variables (0.67 among males and 0.48 among females).

After the transformation the meat consumption structure has changed. Along with reduced red meat consumption the increase in poultry consumption took place (Figure 7). This increase negatively correlated with the pancreatic cancer morbidity (−0.88 among males and −0.57 among females).

Positive trends observed in the recent years can also be a result of the higher fruit consumption compared to previous years (Figure 8). In the years 1990–2008 a high, negative correlation between analyzed variables was observed (−0.62 among males and −0.50 among females).

A decline in the cereals consumption may have been a factor that highly contributed to the increased pancreatic cancer morbidity before 1995 (Figure 9). Almost a complete, negative correlation between these variables was observed (−0.93 among males and −0.91 among females) in the years 1960–1989. Cereals are the most important source of fibre in the typical Polish diet; therefore, fibre content in the diet also fell in this period (Figure 10). It correlated negatively with incidence rates (−0.84 among males and −0.89 among females).

The consumption of products containing folate is considered as one of nutritional factors that decrease pancreatic cancer risk. Despite certain fluctuations, the folate content in a diet in the analyzed period decreased (Figure 11). In the

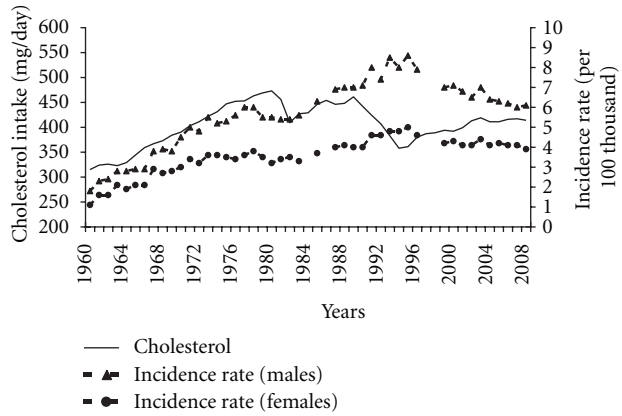

Figure 4: Cholesterol intake and pancreatic cancer morbidity 1960–2008.

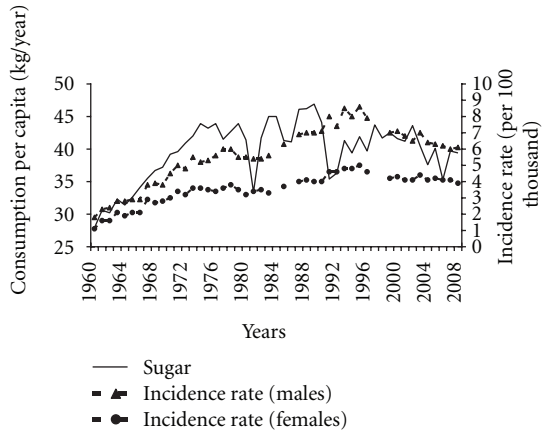

Figure 5: Sugar consumption and pancreatic cancer morbidity 1960–2008.

years 1960–1989 this tendency correlated negatively with the increased pancreatic cancer morbidity (−0.45 among males and −0.49 among females).

The risk of pancreatic cancer may be indirectly boosted by alcohol as a result of chronic pancreatitis. Alcohol consumption was growing visibly between 1960 and 1980 (Figure 12). In the early 1980s its consumption fell to remain at the comparable level in later years. However, since in the early 1980s alcohol began to be rationed in Poland, there is a strong possibility that data on the consumption can be underestimated. Nevertheless, a very high positive correlation between alcohol consumption and incidence was observable in that period (0.86 among males and 0.82 among females). After 2001 another increase in alcohol consumption took place, which has not reversed a positive tendency of reduced pancreatic cancer morbidity so far.

Apart from nutrition, other environmental factors can influence the development of pancreatic cancer, among others, tobacco smoking. The number of cigarettes per person a year was increasing from 1960 to 1979, followed by downward or upward trends lasting a number of years (Figure 13). On the basis of the number of cigarettes smoked

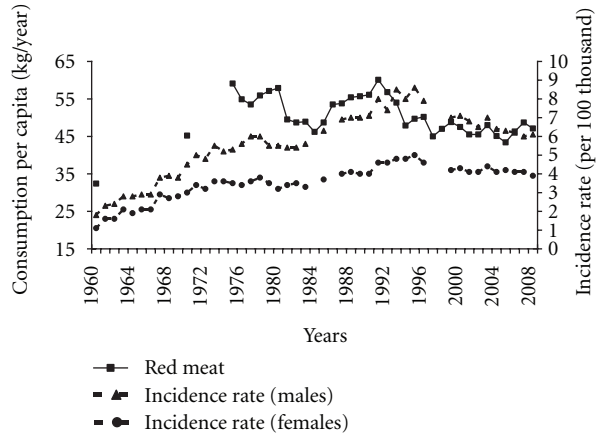

Figure 6: Red meat consumption and pancreatic cancer morbidity 1960–2008.

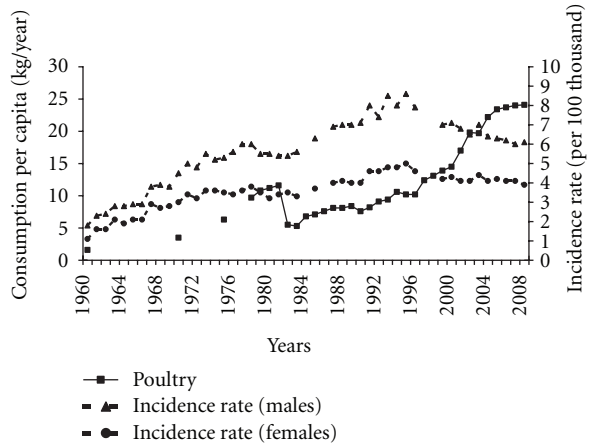

Figure 7: Poultry consumption and pancreatic cancer morbidity 1960–2008.

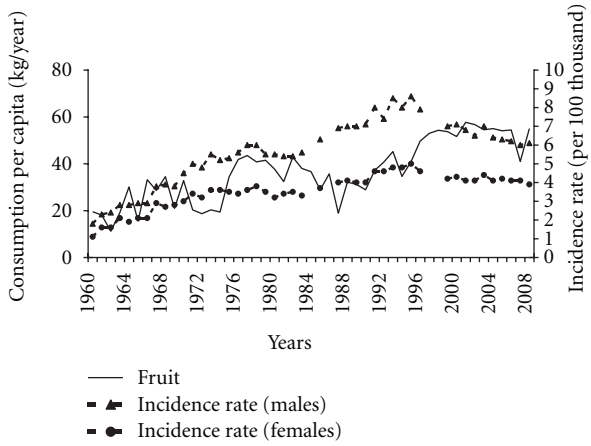

Figure 8: Fruit consumption and pancreatic cancer morbidity 1960–2008.

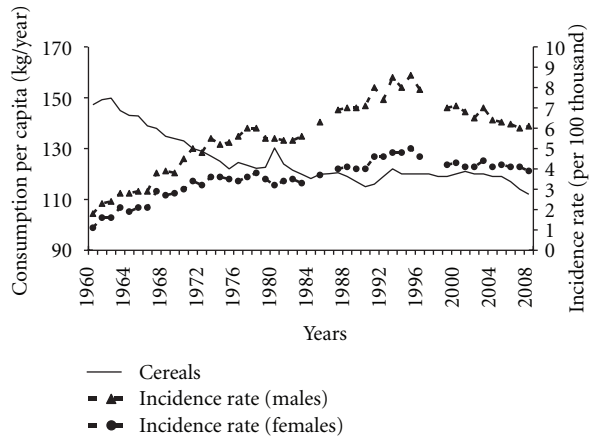

Figure 9: Cereals consumption and pancreatic cancer morbidity 1960–2008.

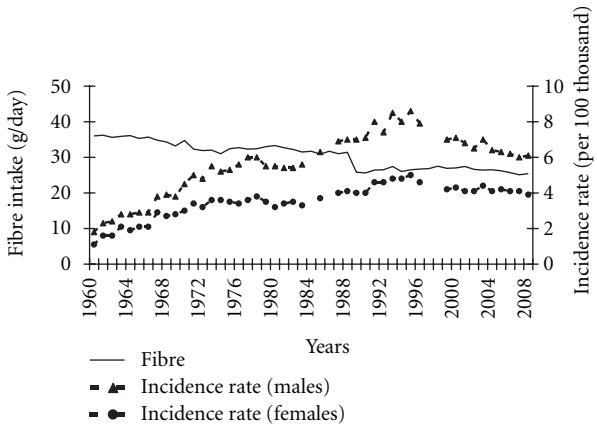

Figure 10: Fibre intake and pancreatic cancer morbidity 1960–2008.

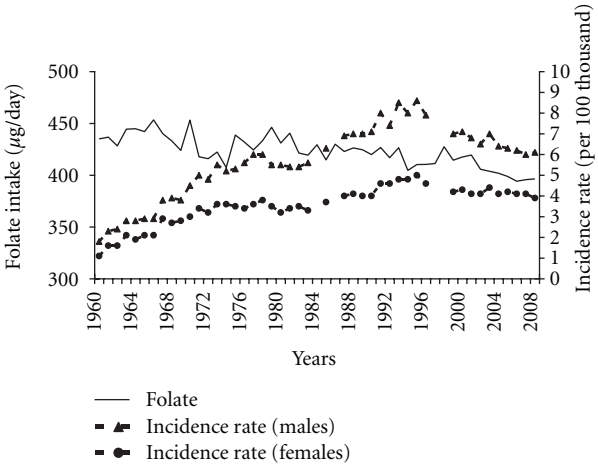

Figure 11: Folate intake and pancreatic cancer morbidity 1960–2008.

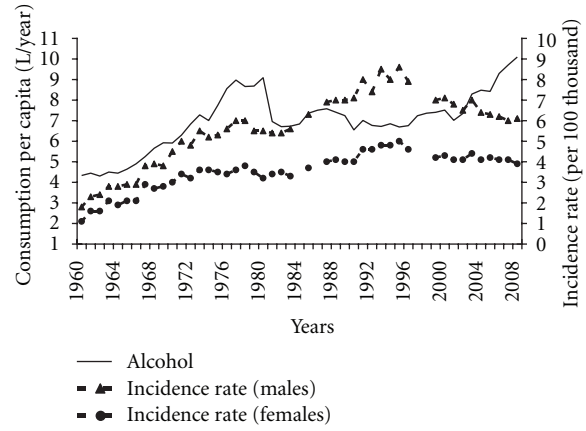

Figure 12: Alcohol consumption and pancreatic cancer morbidity 1960–2008.

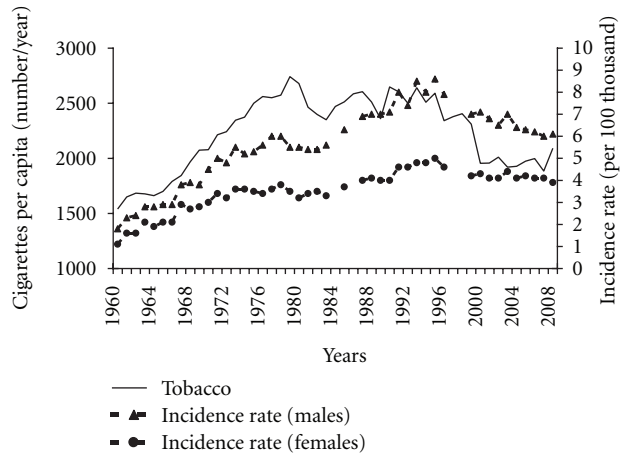

Figure 13: Tobacco smoking and pancreatic cancer morbidity 1960–2008.

and incidence rates, a positive correlation was found in the entire period under analysis (0.55 among males and 0.44 among females). Frequent smoking could contribute to the increased incidence in the beginning. A significant decrease in cigarettes consumption in the years 1995–2000 can be one of the factors that positively influence the observed downward trend as regards pancreatic cancer morbidity.

4. Discussion

It is very difficult to prove the relationship between environmental factors, lifestyle factors such as tobacco smoking, body weight, diet, and cancer. The abovementioned relationship needs to be proven on the basis of epidemiological research [16]. Moreover, the influence of various factors on carcinogenesis needs to be proven on the basis of experimental study.

The risk of pancreatic cancer is probably increased by a number of different factors. One of the best proven risk factors is tobacco smoking [1, 17]. There is not enough evidence

to claim with absolute certainty that there is a relationship between cancer and diet although the negative impact of body fatness, especially abdominal fatness, excessive energy intake, high consumption of fat, red meat, and sugar is frequently taken into consideration. On the other hand, high consumption of fruit, products containing dietary fibre, folate, and other vitamins can probably reduce the risk [18].

High energy intake in Poland up to 1989 was correlated with pancreatic cancer incidence. In this case, oxygen-free radicals, which cause DNA damage to cells, play a very important role in the process of carcinogenesis [19, 20]. Excessive energy content in a diet can also lead to overweight and obesity. Studies show that over a half of adult Poles are overweight or obese, and very often this obesity is abdominal one [21, 22]. Unfortunately, no long-term, national, or systematically repeated surveys were carried out in this field in Poland, the results of which would allow an establishment of statistical correlation.

Until the transformation period, the high energy intake was related to excessive consumption of fats, cholesterol-rich products, and sugar, which evidently correlated with the growing pancreatic cancer incidence.

There are some mechanisms that can explain an association between high fat consumption and pancreatic cancer risk. When fat gets into the duodenum, cholecystokinin is released [23]. That stimulates lipases secretion by pancreas. However, when high amounts of fatty acids found their way to duodenum for a long time, pancreatic hypertrophy or hyperplasia may take place. Then pancreas is more prone to adverse activity of carcinogens. Moreover, high content of certain fatty acids in a diet can contribute to the secretion of bile acids to the pancreatic duct [24]. Bile acids can stimulate cyclooxygenase-2 (COX-2) release and promote carcinogenesis.

Excessive energy intake and high content of saturated fat and sugar in a diet are associated with insulin resistance [25–27] and diabetes that can increase the risk of pancreatic cancer [28]. Prolonged hyperinsulinemia increases endogenous levels of insulin-like growth factor-1 (IGF-1) [29]. Both insulin and IGF-1 affect the increase in abnormal cell proliferation and reduction of apoptosis.

The influence of fat and cholesterol content in a diet on the development of pancreatic cancer is not definite. The data from a population-based case-control study conducted in California provided some evidence that fat and cholesterol may increase the risk of pancreatic cancer [30]. Some analyses indicate the adverse activity of animal fats in particular [31, 32]. On the other hand the authors of the Multiethnic Cohort Study in Hawaii found no associations of pancreatic cancer risk with intake of total fat, saturated fat, or cholesterol [33].

Results of our study demonstrate that pancreatic cancer incidence in Poland could be more linked to animal fats consumption than to total fats consumption. After 1995 the incidence fell despite the growing total fats consumption. This is related to beneficial changes in the fat consumption structure, as the share of animal fats radically fell in favour of the consumption of vegetable fats.

Surveys also confirm the adverse influence of high sugar consumption. According to the abovementioned study from Hawaii, the risk of pancreatic cancer increased with higher intake of total sugars, fructose, and sucrose [34]. Also the results of a prospective study conducted in Sweden confirmed that the high consumption of sugar and high-sugar foods might be associated with a greater risk of pancreatic cancer [35].

Another factor that can possibly contribute to the pancreatic cancer incidence is red meat. It seems that the decline in consumption of this food after 1989 had a critical significance to the downward trend. Red meat is a source of heme iron, and free iron can favour the creation of free radicals [1]. The method of preparing dishes can also be important in this respect [1, 36]. Cooking at high temperatures (frying or grilling) produces heterocyclic amines and polycyclic aromatic hydrocarbons that pose a potential risk of cancer.

Many surveys confirm the relationship between red meat consumption and increased risk of pancreatic cancer, but not all of them. Recently published results of meta-analysis of prospective studies indicated that red meat consumption was positively associated with pancreatic cancer risk in men but not in women [37]. On the other hand, according to the other meta-analysis, red meat was associated with higher pancreatic cancer risk in case-control studies but not in cohort studies [38]. Results from the large population-based prospective cohort of women in Sweden support a possible positive association of long-term red meat consumption and an inverse association of long-term poultry consumption with pancreatic cancer risk [36]. Since many surveys have not confirmed the relation between the poultry consumption and the risk of pancreatic cancer [33, 39, 40], the authors of Swedish survey believe that their observations may be attributable to chance [36]. Our analysis also attributes reduced morbidity to the increased poultry consumption; it seems though that, above all, it was a result of the changing meat consumption structure.

Fruit can protect pancreas against cancer. They are a source of vitamin C and other antioxidants; they have the ability to trap free radicals and reactive oxygen molecules, protecting against oxidation damages [1]. Moreover, flavonoids found in fruit inhibit the metabolic activation of carcinogens by cytochrome P450 enzymes or by detoxifying and cellular defensive enzymes [1, 41].

Some surveys confirm a positive influence of fruit as regards protection against pancreatic cancer [38, 42], and others do not observe such a link [43]. In Poland, the growth in fruit consumption may have contributed to the reduced incidence in recent years.

Reduced risk of pancreatic cancer may be related to the consumption of fibre-rich products, including cereals, particularly wholegrain ones. Potential protective influence of fibre intake on pancreatic cancer could be explained through association with insulin resistance, triglyceride, and high density lipoprotein (HDL) level [42]. Some studies support the hypothesis that consuming more whole-grain or high-fibre foods may reduce the risk of pancreatic cancer [42, 44].

In Poland, the decline in consumption of products containing fibre, including cereals, has probably increased the risk of pancreatic cancer.

Food containing folate is one of the factors reducing the risk of pancreatic cancer. It is assumed that folate influences carcinogenesis through its role in methylation reactions, nucleotide synthesis, and DNA repair [1, 6]. The authors of the population-based prospective study in Sweden found a strong inverse association between the dietary folate intake and the risk of pancreatic cancer [45]. However, folic acid from supplements did not show a protective effect. Other surveys have also confirmed the positive impact of folate [46, 47] on the prevention of pancreatic cancer, although not all of them [48, 49].

In Poland we have observed a decreasing folate content in a diet for many years. It seems that this fact had more significance before the transformation, where other nutritional aspects also influenced the pancreatic cancer morbidity.

Alcohol is not considered a direct risk factor for pancreatic cancer [7, 8]. However, chronic alcohol drinking can cause pancreatitis whilst heavy alcohol consumption leads to diabetes mellitus. These two diseases are risk factors for pancreatic cancer. Moreover its metabolites, such as acetaldehyde and fatty acid ethyl esters, can modify metabolic pathways involved in the inflammatory response and carcinogenesis. A hospital-based case-control study in Texas has shown that heavy alcohol consumption (>60 mL ethanol/day) significantly increased pancreatic cancer risk [50]. According to a population-based case-control study in different US towns, alcohol drinking at levels typically consumed by the general population did not appear to be a risk factor for pancreatic cancer although heavy consumption could be related to risk [51].

The consumption of alcohol in Poland was growing until 1980 and could be one of the factors favouring the pancreatic cancer morbidity. The introduction of alcohol sale rationing in the 1980s contributed to the reduced consumption however, these data may be underestimated. It is hard to definitely state that this fact stood in any relation to the future reduction in incidence rates. The recently observed increased alcohol consumption has not had a negative impact on the incidence. Taking into account the indirect influence of alcohol on pancreatic cancer, we cannot rule out that its adverse effects will manifest themselves later on.

Tobacco smoking is the strongest environmental risk factor of pancreatic cancer causing 20–25% of all pancreatic tumours [2, 52]. It is most probably connected with the mutagenic effect of the tobacco smoke components such as heterocyclic amines and polycyclic aromatic hydrocarbons (PAH) on protooncogenes in cells, which cause especially K-ras mutations [53]. It is an important early stage of carcinogenesis. After PAH enter the organism, they are metabolized by detoxifying enzymes into forms capable of interacting with DNA. As a result of the activity of the P450 enzyme system (CYP1A and CYP1B), active epoxy compounds are formed. These are then hydrolyzed by the epoxy hydrolase into diol epoxide derivatives. They can fix with DNA (in the position of N2 glutamine), and this, in turn, can lead to P53 gene mutation. The high prevalence of K-ras mutations in smokers and drinkers with pancreatic cancer might reflect combined carcinogenic effects of tobacco and alcohol [54]. Additionally, smoking is an independent risk factor for chronic pancreatitis [7, 53]. Giving up smoking substantially reduces the future incidence of pancreatic cancer [55].

In Poland for many years tobacco smoking was increasing and then remained at a high level. That was accompanied by an increasing incidence of pancreatic cancer. Later, as the number of cigarettes smoked was falling, a substantial decrease in pancreatic cancer incidence was observed.

On the basis of research on dietary factors, which may have an impact on pancreatic cancer morbidity in Poland, it has been proven that the majority of observations conducted in other countries also concern Poles. According to the conducted investigations, the factors that increase the risk of developing pancreatic cancer, to different extents, is high consumption of red meat, fats, especially of animal origin, sugar, and cholesterol intake, and the factor that reduces the risk is consumption of fruit, cereals, and, perhaps, also folate and dietary fibre. What is very interesting and important from the point of view of measures to prevent pancreatic cancer is the fact that the growth rate of pancreatic cancer incidence in Poland has been reversed since 1995. This state of affairs is associated with better diet, that is, lower energy intake, along with lower red meat and animal fat consumption and higher fruit consumption. It also seems that positive changes as regards smoking have had a positive impact in the subjective issue.

Pancreatic cancer is a type of cancer that has an especially poor prognosis [1, 2]. Many cases are diagnosed at a late stage of the disease, and this fact makes it impossible to implement any radical surgical treatment. Special attention should therefore be paid to prevent this type of cancer by changing lifestyle habits. As it appears from presented research, tobacco smoking and alcohol consumption should be kept low. Moreover, an educational campaign should be conducted on a wide scale in order to further improve dietary habits.

5. Conclusions

Increase in the pancreatic cancer morbidity in Poland in 1960–1995 was probably related to adverse dietary patterns up to 1989: high energy and cholesterol intake, decrease in fibre and folate content in a daily diet and high consumption of total fats, animal fats, sugar and alcohol, and the decrease in cereals consumption.

Lowering red meat and animal fats consumption as well as increasing fruit consumption after economic transformation could influence incidence reduction observed in 1996–2008.

Changes in tobacco smoking probably also affected pancreatic cancer morbidity. Growing or large number of cigarettes smoked was accompanied by a rise in incidence,

whilst reduced smoking was associated with decreased morbidity.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 585674, 6 pages
doi:10.1155/2012/585674

Clinical Study

Microenvironment Elements Involved in the Development of Pancreatic Cancer Tumor

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Received 29 June 2012; Accepted 22 November 2012

Academic Editor: Grazyna Rydzewska

Introduction. In spite of intensive research during many years, pancreatic adenocarcinoma remains one of the deadliest cancers. The surgical intervention remains main possibility of treatment because chemotherapy and radiotherapy has a minimal impact on long-term survival. We are still looking for the weak points of this devastating disease. *Materials and Methods*. Pancreatic tumor tissue samples were collected from 36 patients. Immunohistochemistry staining was used to evaluate expression of growth factors and immune infiltrates. Activity of MMP2 and MMP9 was assessed by gelatin zymography on 7.5% SDS-PAGE gel with 0.1% gelatin. *Results*. All growth factors were strongly expressed in pancreatic tumor tissue. We found that level of expression of c-Met receptor was higher for G3 tumors than for G2 tumors. Also we found that active MMP2 was present at all stages of tumor while active MMP9 just at more advanced tumors. Abundant immune cells infiltration was distinctive for tumor tissue, especially macrophages were infiltrating tumor tissue. We found that amount of macrophages was associated with lymph nodes metastases. *Conclusion*. In our research we demonstrated that among many factors influencing tumor microenvironment c-Met receptor, infiltrating macrophages and MMP2 have significant influence on development and invasion of pancreatic cancer.

1. Background

In spite of intensive research during many years, pancreatic adenocarcinoma remains one of the deadliest cancers. The surgical intervention remains main possibility of treatment because chemotherapy and radiotherapy has a minimal impact on long-term survival. Research over the last twenty years has yielded much insight into pancreatic cancer biology, but it has neither improved diagnostics methods nor the way of treatment. We are still looking for the weak points of this devastating disease.

It was shown in recent years that the tumor microenvironment plays a critical role in tumor progression [1]. In pancreatic tumors this microenvironment is particularly heterogeneous. It consists of dense fibrotic stroma with cancer cells, stellate cells, infiltrating inflammatory cells, and the remains of the proper structure of the pancreas. These cells are the source of various growth factors as well as proangiogenic factors. The fibroblasts present in the tumor's tissue are responsible for the production of collagen and fibronectin which increase the chemoresistance of the tumor. Because of abundant fibrotic tissue, the tumor environment is strongly hypoxic [2, 3]. All these factors contribute to the disease's aggressive nature and occurrence of early metastases.

Infiltrating inflammatory cells are a rich source of factors influencing tumor growth, invasion, and metastases. Macrophages are of particular interest. Their role in the tumor's environment has been studied in recent years [4]. It is known that they are a rich source of growth factors that stimulate cell proliferation like EGF, PDGF-BB, HGFα, and TGF β. Furthermore they also produce matrix metalloproteinase-9 (MMP9) which takes part in various essential processes [5].

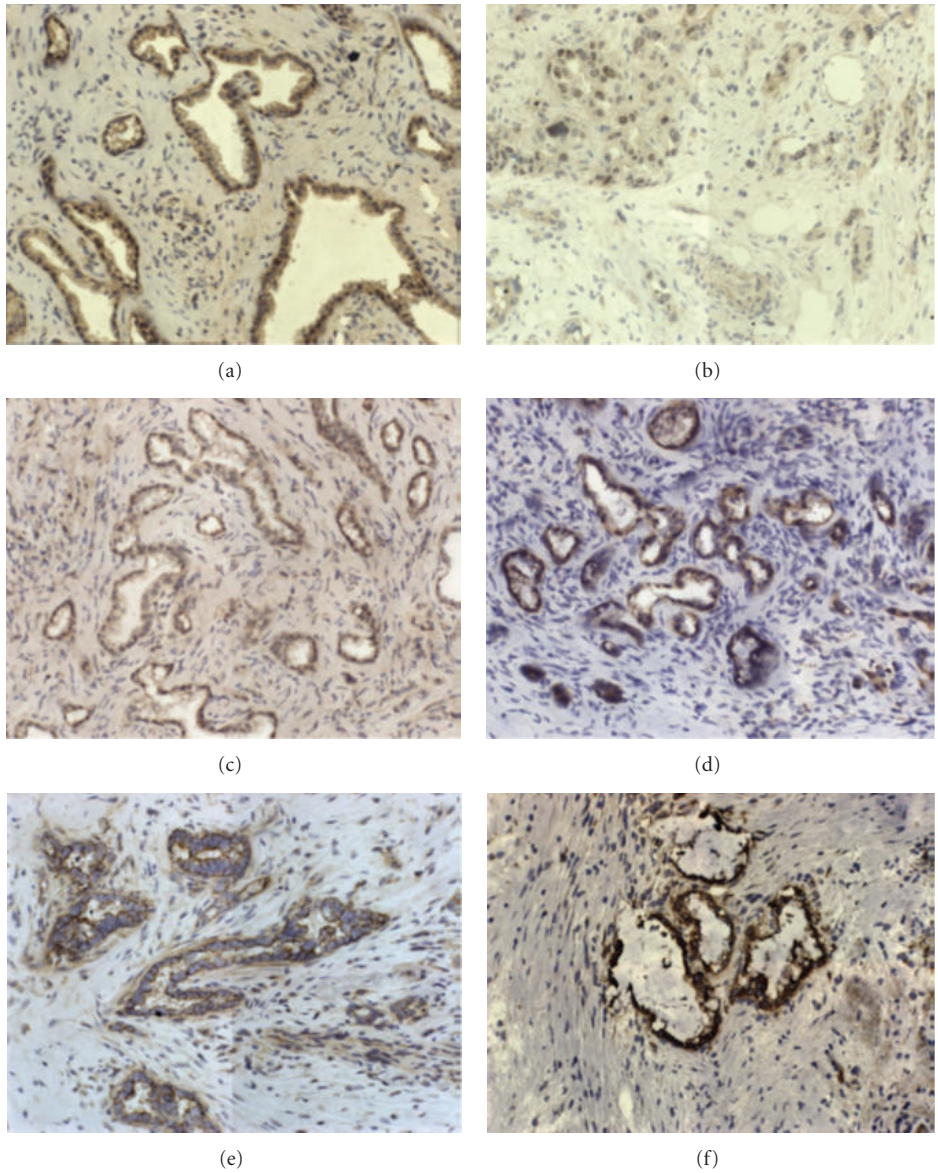

Figure 1: Expression of growth factors: (a) PDGF-BB cancer nests, (b) PDGF-BB stroma, (c) EGF, (d) EGFR, (e) HGFα, (f) c-Met. Original magnification $\times 200$.

Matrix metalloproteinases are a group of 20 proteases divided into 4 subclasses: collagenases, gelatinases, stromelysins, and membrane-type MMPs. They take part in modification of extracellular matrix which makes them important component of such processes as angiogenesis, cell migration and metastasis formation. They also participate in activation of many proteins and in this way they can regulate proliferation and apoptosis [6].

MMP 2 and 9 are classified as gelatinases. Their role in metastasis formation is significant because their substrate is collagen IV, a component of basement membrane. Its degradation makes it possible to migrate for cancer cells. Increased expression of MMP 2 and 9 was stated in many types of cancer. In breast cancer [7] their activity was associated with distant metastasis and in case of oesophageal carcinoma with increases in invasiveness [8].

Also in pancreatic cancer strong expression of MMPs was shown. In a research conducted on 6 cell lines, it was shown that increased expression and activity of MMP2 are associated with greater invasive potential of pancreatic cancer cells [9].

The aim of our study was to investigate three points that may have an influence on the development of the tumor: expression of growth factors, inflammatory cells infiltration, and enzymatic activity of matrix metalloproteinases so as to have a more complete picture of the pancreatic cancer tumor.

2. Materials and Method

2.1. Patients and Specimen Collection. Pancreatic tumor tissue samples were collected from 36 patients who underwent surgical resection due to pancreatic cancer. Tissues were

collected based on protocol approved by the Bioethics Committee of Warsaw Medical University. Tumors were classified according to TNM staging and tumor grade. PDAC patients ranged between T1–T4 (T1 ($n = 3$), T2 ($n = 6$), T3 ($n = 25$), and T4 ($n = 2$)), N0 ($n = 13$), N1 ($n = 23$), M0 ($n = 33$), and M1 ($n = 3$) stage. Also the histologic grade was evaluated: G1 ($n = 4$), G2 ($n = 16$), and G3 ($n = 16$).

For protein isolation, the samples were frozen and stored at −20°C until they were used. Samples for immunohistochemical analysis with dimensions of 5 × 5 × 5 mm were frozen for 45 seconds in acetone using dry ice at a temperature of −70°C and stored at −80°C.

2.2. Gel Zymography. Proteins were isolated from tumor tissue using Total Protein Extraction Kit (Millipore, Billerica, USA). 10 μg of total protein isolated from the tissue was diluted with 2 parts of Zymogram sample buffer (Bio-Rad Laboratories, Hercules, USA) and resolved by 7.5% SDS-PAGE containing 0.1% gelatin (Sigma-Aldrich, St. Louis, USA). Following electrophoresis, gels were washed with 2.5% Triton X-100 to remove SDS twice for 30 minutes and incubated in developing buffer (50 mM pH 7.5 TRIS buffer with 5 mM $CaCl_2$ and 0.2 M NaCl) for 20 h at 37°C. Gelatinase activity was visualized by staining the gels with 0.5% Comassie blue for 1 h followed by incubation in the destaining solution (methanol 40%, acetic acid 10%). Afterwards the destained gel was rinsed with water. For semiquantitative evaluation, a photo of gel was taken and MicroImage (Olympus, Japan) software was used.

2.3. Immunohistochemistry. Frozen tissue from pancreatic cancer was cryocut into 5 μm sections. Each tissue was stained with hematoxylin-eosin (H&E). The Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse were used for immunostaining. After being dried in room temperature, the slides were fixed with acetone for 10 min. Then they were incubated for 5 min Dual Endogenous Enzyme Block (Dako, Glostrup, Denmark). The sections were incubated with a proper antibody for 25 minutes for EGF (Z-19), EGFR (EGF-R2), PDGF-BB (N-30), HGFα (H-145), and c-Met (C-12) (all form Santa Cruz Biotechnology, Santa Cruz, USA) and for CD68 and CD3 (EBM11; F7.2.38, Dako, Glostrup, Denmark). Afterwards incubation with Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) for 25 minutes at room temperature was followed by a color reaction using Dako REAL DAB+ chromogen for 3 minutes. The slides were counterstained with Mayer's hematoxylin.

2.4. Semi-Quantitative Analysis of Immunohistochemical Staining. For quantitative evaluation, 5 areas were chosen after scanning the tumors sections at low power 40x. These fields were analyzed at 200x magnification using MicroImage software (Olympus, Japan), counting the total stained area.

2.5. Statistical Analysis. A comparison was made: for two groups with the Mann-Whitney U test and for three groups with Kruskal-Wallis Test. The minimal level of significance was defined as $P < 0.05$.

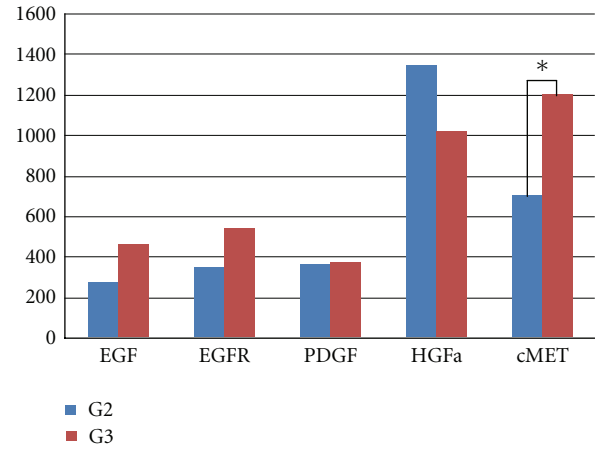

Figure 2: Comparison of expression of growth factors idn G2 and G3 tumors.

3. Results

3.1. Growth Factors in Pancreatic Cancer. 25 tumor tissues were studied immunohistochemically for expression of growth factors in tumor tissue (Figure 1). Expression of growth factors was found in all cases. The immunoreactivity of EGF was weak to moderate in cytoplasm of cancer cells. As for EGFR we found its expression to be moderate to strong in cytoplasm of cancer cells and weak in small ductal cells. PDGF-BB immunoreactivity was moderate to strong in cytoplasm of cancer cells and also in 6 cases we found nuclear staining in cancer cells as well as in infiltrating immune cells. Membranous and cytoplasmic staining for HGFα was strong in tumor cells whereas staining of c-Met was moderate to strong.

Expression of growth factors was compared against tumor grading and N stage. Using a semiquantitative estimation of all considered growth factors expression, the result of statistical analysis showed that the only difference in expression between G2 and G3 group was statistically relevant in case of c-Met receptor ($P = 0.033$) (Figure 2).

3.2. Infiltrating Inflammatory Cells. To evaluate cell infiltrates we used monoclonal antibodies against CD68, HLA II, neutrophil elastase, CD3, and CD56. We found numerous lymphocyte and macrophages infiltrations. There was also a strong expression of neutrophil elastase. No NK cells infiltration was observed. Inflammatory cells were present around neoplastic glands and also strongly around nerves infiltrated by cancer cells (Figure 3).

We compared the results due to N stage and we found that the number of macrophages in tumor tissue was significantly higher in the group with metastases to lymph nodes (401) than the in N0 group (167) ($P = 0.0085$) (Figure 4).

3.3. Gel Zymography. Genolytic activity was studied in 30 tissue isolates from pancreatic tumors. Active MMP2 (62 kDa) was present in 88% cases and MMP9 (83 kDa) in

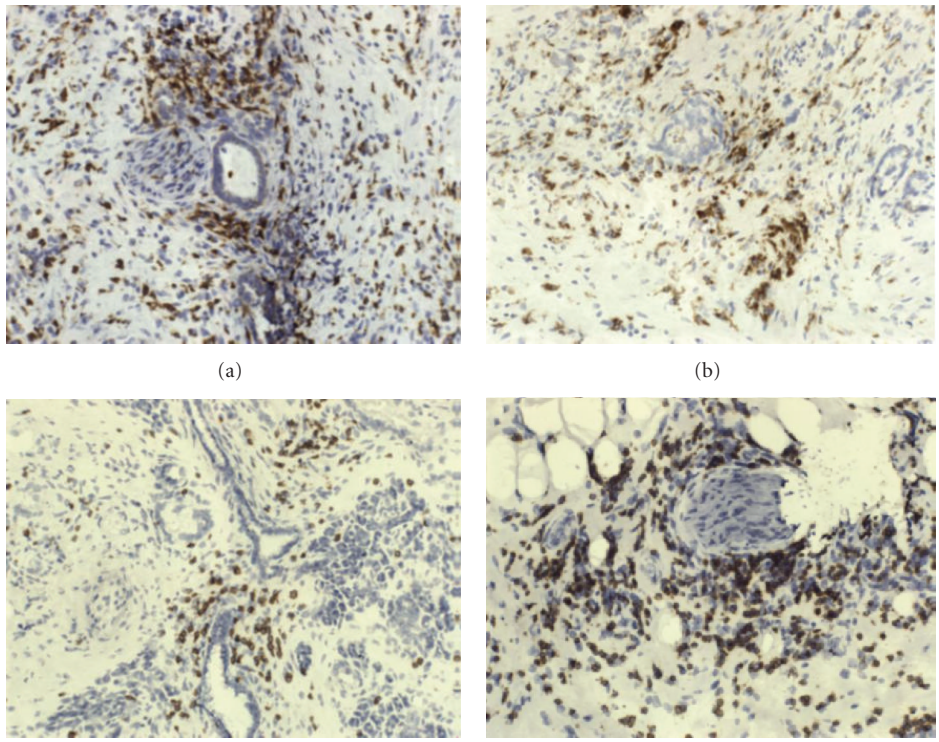

Figure 3: Characterization of the inflammatory infiltrate (a) and (b) CD68 macrophages (c) and (d) CD3: original magnification ×200.

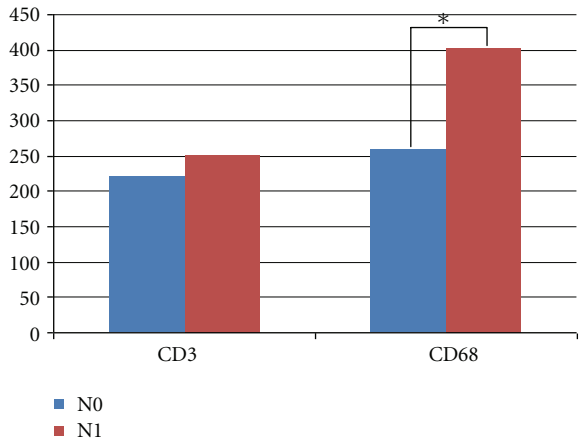

Figure 4: Comparison of inflammatory infiltrates.

38% cases. For 6 samples we were not able to determine MMP's activity because of indistinct picture of gel.

Comparing the results according to histologic trading we can tell that for G1 tumors we did not observe activity of matrix metalloproteinase 9. For G2 tumors active MMP9 was present in 7 ($n = 9$) cases and for G3 only for 4 ($n = 11$). Appearance of active MMP2 was claimed for 3 G1 cases ($n = 4$), in all samples for G2 and for 10 G3 cases (z 11).

Densitometric measurement also confirmed that for well-differentiated tumors matrix metalloproteinases' activity is lower than for G2 and G3 ($P < 0.05$). Activity of MMP2 was, respectively, G1: 3.27 ± 3.6; G2: 16.57 ± 13.9; G3: 13.6 ± 12.2 ($P < 0.05$). Activity of MMP9 was not reported for G1 tumors, and for the other groups it was, respectively, G2: 18 ± 13.9; G3: 38.2 ± 22.3.

4. Discussion

In pancreatic tumors we observed intensive immune cells infiltration. In pancreatic cancer it was reported previously that macrophages are involved in angiogenesis [10], supporting tumor growth and invasion of cancer cells. They are the source of angiogenic factors like VEGF and also MMP9 which degrade extracellular matrix. Tdhe important fact is that macrophages can suppress T cell response. Thus, macrophages infiltrating pancreatic tumor are an important factor in creating metastases. We found that the number of macrophages is higher in the group with lymph node metastases. This observation supports the statement about participation of macrophages in creating metastases.

Abundant expression of growth factors is typical for pancreatic cancer tissue. As they participate in signaling pathways mediated by receptor tyrosine kinases, they might regulate cell proliferation, migration, and survival. Previous studies on expression of growth factors provided and interesting observation. It was reported that a high level of EGF and EGFR correlated with lymph node involvement and distant metastasis as well as reduced median survival [11]. Only in case of PDGF-BB high levels in blood serum were favorable in prognosis [12] and high expression in tumor was

related to decreased pancreatic cancer growth [13]. In our research we observed a strong expression of growth factors; however, only for c-Met receptor we were able to claim a statistically significant difference between G2 and G3 groups. No significant difference was observed in relation to lymph node metastases.

Our results indicate that c-Met might be a pivotal element in pancreatic cancer. This receptor tyrosine kinase is activated by HGF and physiologically it participates in embryonic development and also during adult life in liver regeneration and wound healing [14]. However its overexpression is observed in pancreatic cancer, even in the early stage of carcinogenesis [15].

c-Met activation leads to increased proliferation, enhanced motility, and invasion. Therefore we presume that an increase in c-Met expression between G2 and G3 stages indicates a more aggressive phenotype which might be related to epithelial-mesenchymal transition. EMT is often considered a first step to metastases as it is related to reduced E-cadherin expression and appearance on N-cadherin [16].

Another factor was linked to lymph nodes involvement—presence of macrophages—this was also confirmed by our studies. Our results indicate that the amount of macrophages is significantly higher in the group with lymph node metastases, as it was reported previously [17]. Recently it has been shown that macrophages also participate in EMT process [18]. They not only express EMT-inducing cytokines but also MMP9 that cleaves E-cadherin/β-catenin complex. Tan et al. suggested that macrophage MMP9 supports EMT also by disrupting of basement membrane. This is a crucial mechanism for invasion of cancer cells to other tissues.

Our analysis of activity of MMPs indicates that MMP2 has a greater impact on development of pancreatic cancer. Its activity can be observed even in the early stages of tumor. On the contrary activity of MMP9 was observed only in G2 and G3 groups. MMP2 was previously reported to increase ability of cancer cells to migrate [19] and determination of MMP2 activity in pancreatic juice was told to be useful in diagnosing pancreatic cancer [20]. MMP9 was associated with metastasis and angiogenesis [21]. Lack of its activity in G1 group might indicate that it is triggered in subsequent stage of the tumor.

5. Conclusion

In our research we demonstrated that among many factors influencing tumor microenvironment c-Met receptor, infiltrating macrophages*w* and MMP2 have a significant influence on development and invasion of pancreatic cancer. They all might contribute to EMT and we intend to examine this in our further research.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The would like to thank professor Anna Nasierowska-Guttmejer and Dr. Dorota Łącka for histopathological evaluation of tumors. This study was carried out with support from the Ministry of Science and Higher Education Grant no. NN404/0693/33.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 908963, 7 pages
doi:10.1155/2012/908963

Clinical Study

Percutaneous Fine Needle Biopsy in Pancreatic Tumors: A Study of 42 Cases

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Received 29 June 2012; Revised 19 November 2012; Accepted 22 November 2012

Academic Editor: Grazyna Rydzewska

The technological progress within the range of methods of pancreas imaging and their more common accessibility selects a group of patients requiring a microscopic diagnosis. Percutaneous fine needle aspiration biopsy under the control of ultrasonography (PCFNA/USG) is the method commonly used in determining the character of a focal pancreatic lesion. *Aim of the Work*. An assessment of the accessibility of PCFNA biopsy in the assessment of solid and cystic changes in a pancreas and the correlation of the results of imaging examination, cytological smear and concentration of a serous marker CA19-9. *Material and Methodology*. In our material we analysed 43 cases of tumors of the pancreas among the patients who were at the average age of 59 ± 10.4 (14 women, 28 men) diagnosed by PCFNA biopsy. *Results*. In a group we are 23 cases of cancer, 12 cases of inflammation and 7 cases of cellular atypia for which 2 cases of IPMN were included. The sensitivity of the method was 92.5% but specificity was 68%. In our opinion PCFNA/USG is a method of the comparable sensitivity and specificity with fine needle aspiration biopsy with EUS control and its efficiency depends to a considerable degree on experience and interdisciplinary collaboration.

1. Introduction

Tumors of the pancreas are a difficult problem of contemporary medicine. They are very often diagnosed in the advanced stage because after they reach the right size, they cause clinical symptoms, such as, stomachache, backache, yellowish discoloration of integument or nausea, and vomits. At the time of growing accessibility to modern radiological imaging techniques often and often are found non-characteristic, often small focal lesions which require the further diagnostic. This allows selecting a group of patients with more often recognized precancerous changes or early stages of pancreatic cancer without clinical symptoms of the disease. The first examination which is done most often through percutaneous ultrasound examination of the abdominal cavity (USG), in which the accuracy in defining the character of focal lesions of the pancreas is 50–70%. The accuracy of this method is improved by applying endoscopic USG (EUS), the Doppler method, USG 3D, and intraoperational USG. During the ultrasound examination both percutaneous and endoscopic we can additionally perform fine needle aspiration biopsy (FNA/USG) of a tumor of the pancreas receiving material for cytological examination [1, 2].

According to the character in imaging examinations, focal lesions of the pancreas is divided into solid and cystic tumor. In both groups there are inflammation processes, benign neoplasms, and malignant neoplasms. Because of difficulties in defining the kind of lesions we deal with, only the correlation of clinical symptoms, the result of the imaging examination, cytological or/and histopathological, and the concentration of neoplastic markers manage with some possibility to indicate the type of the lesions. It influences the way of the further therapeutic management.

For the defined group of patients with the clinically unnoticeable as well as the evident tumorous lesions, using this technique in order to get material for a cytological

examination is quite a simple solution. PCFNA biopsy is a method which is safe, cheap, and demonstrates big sensitivity and specificity concerning both solid and cystic tumors under the condition that it is performed in an interdisciplinary way [3].

The aim of the study was to assess accessibility of PCFNA biopsy in the assessment of solid and cystic changes in the pancreas and the correlation of the results of imaging experiments, cytological smear, and concentration of a serous marker CA19-9.

2. Material and Methodology

The research was conducted in a forward-looking way between 2009 and 2011. The analysed a group consisted of 43 patients with focal lesions in the pancreas diagnosed in the Clinical Ward of General, Oncological and Endocrinological Surgery of the Joint Provincial Hospital in Kielce. Patients with the focal lesions of the pancreas recognized in the USG and CT (computed tomography) examination of the abdominal cavity were qualified for the research.

Percutaneous ultrasonography of the abdomen was performed based on the clinical symptoms. Jaundice, gastric obstruction, abdominal or back pain, and weight loss were the main symptoms which were reported by patients. Most patients suffered from fatigue. Jaundice, nausea, and vomiting were common among patients with a tumor localized in the head of pancreas (33 pts), while abdominal or back pain and weakness were present in patients with a tumor of the body of pancreas (9 pts). Some patients had a palpable mass in the abdominal cavity. USG was performed as first diagnostic imaging.

Patients with typical clinical symptoms and imaging changes of inflammation and postinflammation processes of the pancreas were excluded from the analysis. Before performing PCFNA/USG, the patients had percutaneous ultrasonographic examinations as well as CT of the abdominal cavity with a contrast agent performed. Most of the patients had the determined concentration of the CA19-9 antigen in blood serum. The decision of performing PCFNA biopsy/USG was undertaken based on the results of the imaging examinations, qualifying for a biopsy the tumors: solid, cystic with tissue echoes, cystic with dividing walls and calcification. Before performing FNA biopsy the patient's consent to examination was received.

The team which consisted of a radiologist, a pathologist, and a surgeon performed a puncture under the control of USG model ESATOE My Lab Classic C making use of the dynamic character of an examination, the possibility of visualization of blood vessels in the vicinity of the tumor and the possibility of correction of the route of a needle and defining the localization of the end of a needle in the lesion. The needles 22G or 25G of the length of 9 cm were used depending on the conditions of reaching the tumor (the depth of the lesion localization, its size, and echogenicity, the thickness of abdominal integument and visceral lipomatosis). The part of the lesions, mainly cystic ones, underwent another puncture depending on the kind of a tumor and aspirated material. Routinely solid tumors were diagnosed with a needle 22G but cystic tumors were aspirated twice with a needle 25G (low risk of post biopsy fistulas) because according of the kind of liquid the additional material was taken from the intra-cystic tissue echos. Smears on the basic small pieces of glass were fixed in 96% of ethanol and then dyed with hematoxylin and eosin, in some cases with mucicarmine to check the presence of acid *mucopolysaccharides* or cell blocks and immunohistochemistry.

The cytological lesions were grouped in three categories:

(1) *Cancer*: the evident cytological characteristics of a cancer.

(2) *Atypia and inflammation*: among the exponents of inflammation there are cellular populations with atypia in a degree from the small one to the big one without unequivocal characteristics of a cancer.

(3) *Inflammation*: inflammation without the presence of the atypical cells.

The results of the imaging examinations, the concentration of neoplastic markers, and the cytological research were compared to each other in order to define the relationship between them. The statistical analysis was carried out based on the STATISTICA software with the Anova Kruskal-Wallis's test and Spearman's rank correlation.

3. The Result of the Study

In the examined and analysed group there were 14 (33.3%) women and 28 (66.6%) men at the age of 36 to 82 (Figure 1). The average age of the patients was 59 years. The tumors were placed in the head of the pancreas (33 patients—78.5%) and the body of the pancreas (9 patients—21.5%) (Figure 2). The average size of the focal lesions was 2.5 ± 0.9 cm. The diagnostics material during PCFNA biopsy was taken in 42 cases. In one case because of the considerable obesity and visceral adipositas there was no visualization of a needle in the lesion and the examination was regarded as nondiagnostic.

12 inflammations, 7 lesions with atypia and inflammation, and 23 cancers were diagnosed on the basis of FNA/USG biopsy examinations. Table 1 shows the characteristics of the patients with taking into consideration the age and sex of the patients and the type of tumorous lesion.

Generally the average age of the patients in the examined group is 59 ± 10.4 years old but with a group with inflammation 51 ± 9.8 years old (Figure 3); in a group with atypia and inflammation 57 ± 10.6 years old (Figure 4) and in a group with a cancer 64 ± 8.5 years old (Figure 5).

The analysis of Spearman's correlation of the sex with the tumor localization shows the considerable dependence of female with the localization of the tumor in the body of the pancreas $P = 0.0160$ (Figure 6). The similar dependence was not observed for tumors of the head of the pancreas depending on the sex.

In the 23 cases typical cytological characteristics of the cancer were diagnosed (20 cases of ductal caricinoma, 1 of mucinous carcinoma, and 2 neuroendocrine carcinoma) (Figures 7 and 8). In 12 cases the cytological characteristics

Table 1: Type of tumor, age and sex of patients, and tumor localization.

	Age patients	Sex	Tumor localisation	
			Head	Body
Inflammation	51 ± 9.8	2 female 10 male M/F 5 : 1	12	0
Atypia and inflammation	57 ± 10.6	1 female 6 male M/F 6 : 1	7	0
Cancer	64 ± 8.5	11 female 12 male M/F ≈ 1	14	9 (7 female, 2 male)

Figure 1: Sex of the patients.

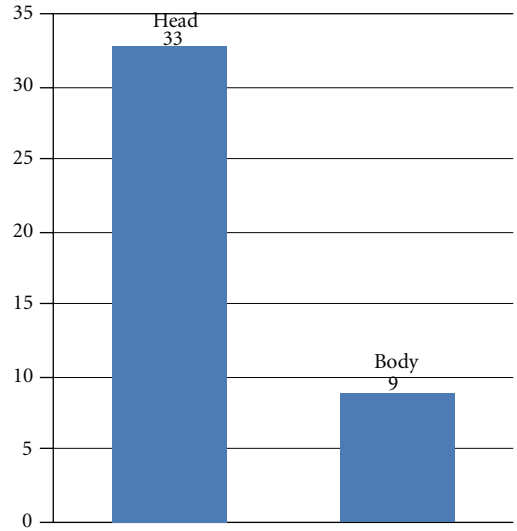

Figure 2: Localisation of the tumor.

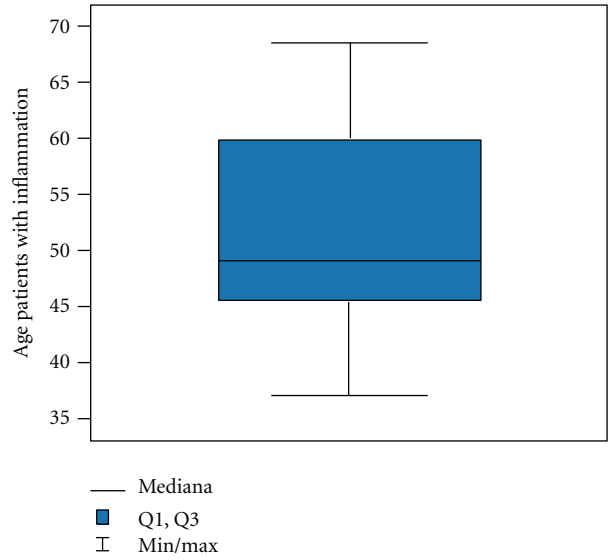

Figure 3: Age in group with inflammation.

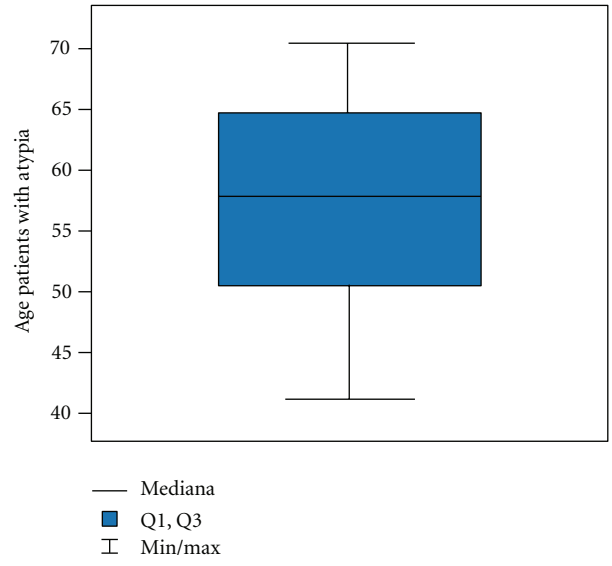

Figure 4: Age in group with atypia.

of inflammation without the presence of epithelial cells with atypia, but in 7 cases they determined the characteristics of inflammation and the presence of atypical epithelial cells (Table 2). In this group, out of 7 cases classified as the atypical lesions there are two cases of IPMN with a dysplasia of a considerable degree without certain cytological exponents of the coexisting cancer (Figures 9 and 10).

Taking into consideration the data from PCFNA biopsy and clinical data, the cancer of the pancreas was finally recognized in 25 patients. In 23 based on PCFNA biopsy and in 2 based on the clinical course of a disease. In 2 people for whom a cancer was diagnosed based on metastasis changes in the liver discovered in the imaging examinations based on PCFNA biopsy, inflammatory lesions with atypia were stated. In this group there are both cases of IPMN.

Table 2: Cytological results.

	n	True positive (TP)	False negative (FN)
Cancer	23 20 ductal carcinoma 1 mucinous carcinoma 2 neuroendocrine carcinoma	23	0
Atypia and inflammation	7	21 (2 IPMN)	2 (1 IPMN with hight grade atypia and ductal carcinoma and 1 ductal carcinoma)
Inflammation	12	12	0

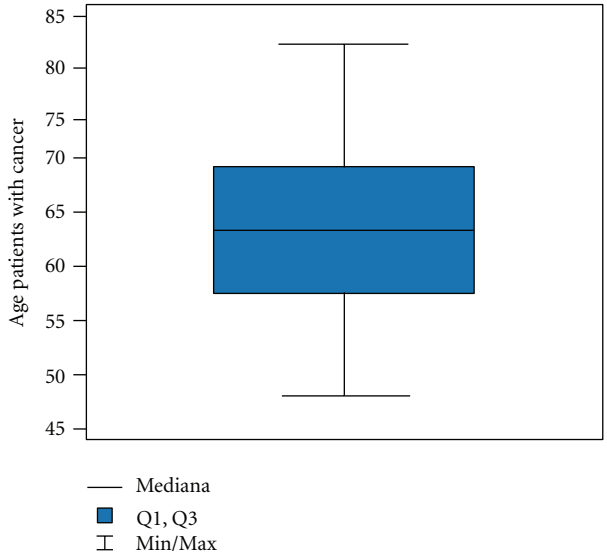

Figure 5: Age in group with cancer.

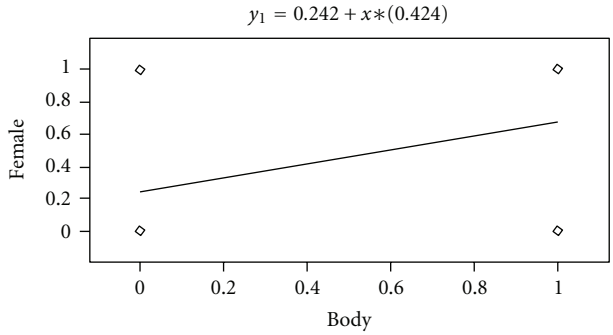

Figure 6: Linear relationship body localisation of tumor with female.

Among 23 patients with a cancer diagnosed on the basis of PCFNA biopsy/USG examinations there were not any removals of a section of an organ.

All the cancers were classified according to cTNM (clinical TNM) classification. Preoperative imaging, upper gastrointestinal endoscopy, and serum concentration of CA 19-9 provided necessary information of clinical advancement of the disease. In some cases, additional information was provided by endoscopic retrograde cholangiopancreatography (ERCP).

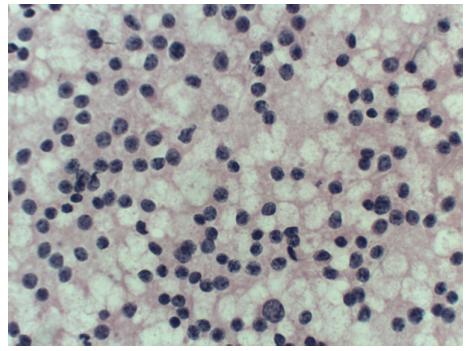

Figure 7: Neuroendocrine carcinoma (typical "salt and peper" chromatin). H-E 40X.

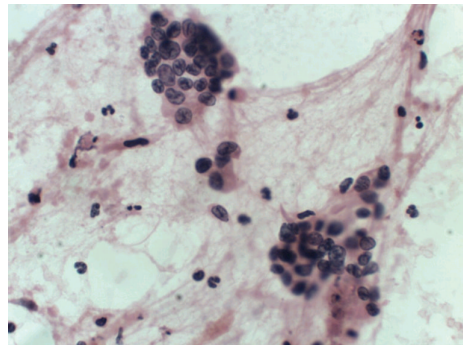

Figure 8: Well-differentiated ductal carcinoma H-E 20X.

The lack of possibility of radical treatment was stated on the basis of the results of the imaging examinations in 11 patients (the characteristic T3/T4 and M1), ERCP in 2 patients, and laparotomy in 9 patients. The characteristic which disqualified the patients from treatment with the use of resection were metastases in the liver or peritoneum, infiltration of the tumor on a superior mesenteric vessels, or a portal vein. The removal of a section of an organ was performed in one case when inflammation with atypia was recognized where based on PCFNA biopsy/USG and in a

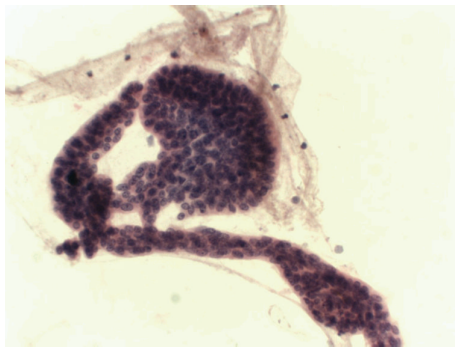

Figure 9: IPMN with low-grade dysplasia. Regular sheet columnar epithelium and mucoid background H-E 20X.

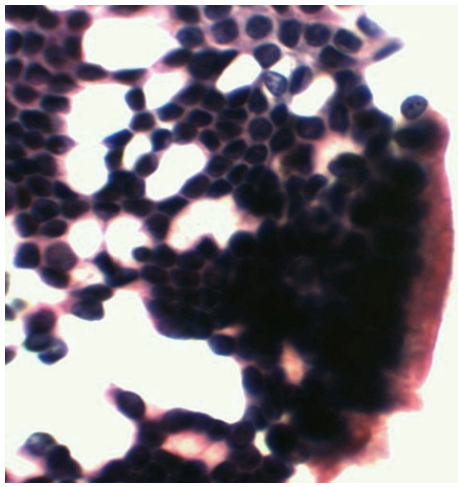

Figure 10: IPMN with high-grade dysplasia. Cribriforme epithelial hyperplasia. Mucikarmin 40X.

postsurgical examination weaving of adenocarcinoma was stated.

Palliative procedures, a double bypass was performed when a laparotomy was necessary to evaluate unresectability of the tumor. Biliointestinal anastomoses in the 3 cases and gastrointestinal anastomoses in the 9 cases were performed when it was possible. In the 2 cases, endoscopic biliary drainage was performed.

The assessment of the analysis of sensitivity and specificity of the results depends closely on the classifications of cytological lesions depicted as "with atypia". Assuming that cytological atypia is treated as a positive examination, the sensitivity was 75% and specificity was 29%. Because of the fact that cytological atypia is not connected with determining recognition and then purposeful treatment, in the research we acknowledged changes with cytological atypia as the negative results although we were aware of two cases of IPMN and one case of a cancer. For such a formulated rule the sensitivity was 92.5% but the specificity 68%.

The analysis of the result of the cytological research with a USG/CT image shows a close correlation at the level $P < 0.0001$ of unequivocal recognition of a cancer with a solid tumor, hypoechogenic in the USG examination. The cystic tumor with tissue echoes in the USG examination shows a considerable correlation with cytological lesions depicted as unspecified atypia, including 2 cases of IPMN $P = 0.00017$.

The inflammatory lesions appeared only in the head of the pancreas with the coincident frequency among women and men and in the correlation of the USG image (cystic tumors without solid mural parts, periductal hypoechogenic signs)showed the similar correlation for each sex ($P = 0.035$).

In the group with a cancer the average value of the concentration CA 19-9 was 1082 ± 1526 U/mL; in the group with atypia was 973 ± 1631 U/mL, but in the group of inflammation was 20.6 ± 26 U/mL. There was not a considerable difference of the value of CA19-9 when the groups with a cancer and atypia were compared but when they were compared with the group with inflammation there was a considerable difference ($P < 0.0001$).

4. Discussion

Among the majority of the patients qualified for pancreas cancer resection evaluated cytological or histopathological procedures with different sensitivity, specificity, and potential complications are conducted. The lack of microscopic sure verification kind of a tumor in the 4–6-week observation and characteristic clinical image as well as lesions in the imaging examinations (USG, CT, NMR, PET) are sufficient factors which prove the need of the resection of the pancreas.

PCFNA biopsy was mostly performed in those patients for whom there was a high possibility of not performing resection of a tumor. This is a method with sensitivity and specificity over 90% for a cancer of the solid type, however specificity lowers considerably for cystic lesions. A core biopsy increases considerably sensitivity and specificity of recognizing solid lesions or parts of cystic ones, however the risk of the complications of percutaneous core biopsy for the parts of lesions in the head and the body of the pancreas is significantly bigger than for FNA and is about 5%. Also the frequency of the complications, namely, postbiopsy pancreatitis, bleeding, pathologic fistulas and infection is considerably bigger during FNA cystic lesions and FNA under the control of EUS [3–10], Laparotomy and core biopsy or the cuneiform resection of a tumor under the control of eyesight characterizes the biggest sensitivity and specificity, however it is the most invasive with a high risk of complications, expensive which requires at least several days of hospitalization.

PCFNA biopsy has a bigger specificity, and at lower frequency in comparison with taking brush swabs during the endoscopic retrograde cholangiopancreatography (ECPW). A brush biopsy is a kind of an examination which enables to identify a group of patients with lesions of dysplastic ductal epithelium like a pancreatic intraepithelial neoplasia (PaIN)s and IPMN), however differentiation between atypia/cytological dysplasia with a regeneration atypia is often very difficult [3, 11, 12].

In the case of the solid tumors of the head and the body of the pancreas, PCFNA biopsy is a method with

a sensitivity and specificity of 90–100% depending on the size and localization of a tumor, a method of biopsy and experience of the team who performed this examination. The kind of tumors which causes diagnostic problems and are the causes of differences in a strategy of dealing with these problems between the environment of gastrologists and surgeons are tumors of the cystic types [13–17].

The aspirated biopsy under the control of EUS is the best tool for the cytological assessment of pancreas carcinoma especially with localization in head. Usefulness this method in tumor of the corpus and tail of pancreas is very limited. Harewood demonstrated the presence of tumorous lesions in the pancreas in 185 patients where the previous biopsies performed in the technique of guiding in CT or during ECPW gave a negative result. PCFNA biopsy/EUS had sensitivity of 94% and thoroughness of 92% for discovering pancreas carcinoma among the patients with diagnostically negative results of the examination of the material taken during ECPW and the sensitivity of 90% and 84% of thoroughness among the patients with negative biopsies under the control of CT [11]. In the examined group the sensitivity of the method was similar although the examination was carried out with the use of percutaneous USG and was 92.5%.

We demonstrated the high sensitivity of the imaging examinations (USG and CT) in the assessment of solid hypoechogenic/hypodense solid lesions. In our material each case like this was treated with the suspicion of a cancer. The radiological changes depicted as a cystic tumor with tissue echoes demonstrated the dependence ($P = 0.00017$) with cytological atypia in smears and included two cases of IPMN.

The criteria of the tackling cystic tumors but not the mucoid ones, which were established in 2006, select the certain group of the cystic tumors which requires the special diagnosis and possible surgical treatment. As indicated for the surgical treatment there are tumors >3 cm with the symptoms or tumors of the size 1–3 cm with high-grade stigmata (dilated main pancreatic duct >6 mm and mural nodule). The risk of the suspicion of a cancer is in a case: marked dilatation pancreatic duct >10 mm, large mural nodule, irregularity of the ductal wall, thickened septum-like structures. [18–20]. The role FNA/USG is discussed with reference to benefits and risks of the method, however but some is used in a routine way and also allows to recognize rare cancers of the pancreas [21, 22]. The aim of differentiating between the inflammatory lesions and tumorous ones includes the assessment of the concentration of CEA, CA19-9, and activity of amylase in the aspirated liquid from the cystic lesion of the pancreas [23]. This is valuable information in the process of differentiating inflammatory lesions and mucous cystic tumors (IPMN). Based on 116 cystic neoplasms Lahat and others determined the lack of the clinical symptoms in 27%, thus small symptoms such as pain and epigastric discomfort in 57% [24]. Pitman in his work pays attention to the specific difficulty in an unequivocal cytological assessment of mucous tumors where dependently on the degree of atypia/dysplasia the specificity is from 30 to 70% [18]. Diagnostic difficulties of the solid lesions with the possibility of the negatively positive results appear in the course of autoimmune pancreatitis (AIP), chronic pancreatitis, and PaIN [14, 25–27].

In 2011 Zubarick proposed to assume the control over the assessment of CA19-9 patients with the family history concerning the carcinoma of the pancreas. In case of the above normal concentration of CA19-9 the patients had FNA/EUS examinations of the indicated focal lesion. In this way neoplastic lesions were diagnosed in 5 patients (0.9% of the analyzed group in which no symptoms were recognized). The diagnosed lesions: 1 neuroendocrine tumour, 1 IPMN, 1 mucous neoplasm, and 1 PaIN. The carcinoma of the pancreas was diagnosed in 1 patient (0.2%) [28]. One can remember that mucous tumors, including IPMN are rarely characterized by significantly above normal CA19-9, and in our material we observed solid carcinoma with CA19-9 within the norm.

There is a worrying clinical course of the tumor of the pancreas. Clinical symptoms were not characteristic, were underestimated by patients and doctors, and late appearance of the tumor of the pancreas was common in the course of the disease. Consequently respectability of pancreatic tumors is very rare. In the group with identification of the carcinoma of the pancreas 13 patients were disqualified from surgical treatment because of the advanced stage of the local tumour (infiltration on blood vessels) or the presence of metastases in the liver. Resection was not carried out in any of the 9 patients who were treated with laparotomy. They limited it to the performing of the drainage procedures. We performed biliointestinal anastomoses in some patients with jaundice and gastrointestinal anastomoses in all patients who have laparotomies. This procedure is recommended as prevention of duodenal occlusion [29]. The late recognition of the pancreas carcinoma and lack of effects of treatment in the depicted group is bigger than in academic literature [6, 11]. It is also important to take into consideration that additionally 4 cases of the pancreas carcinoma were recognized during observation of patients, in a cytological examination inflammation with atypia were stated but a clinical course might be appropriate to the carcinoma of the pancreas.

5. Conclusions

(1) PCFNA biopsy/USG is in connection with the imaging examinations, a sensitive and safe method of recognizing the advanced stage of the carcinoma of the pancreas.

(2) The results of cytological inflammatory lesions with atypia should pay a special attention to those patients as the potential candidates for surgical treatment as for possibility of coexisting of the carcinoma of the pancreas.

(3) In the diagnosing of the carcinoma of the pancreas also other clinical data such as the concentration of CA19-9, the ultrasonographic image of the focal lesion of the pancreas, and their localization may be helpful.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 243524, 7 pages
doi:10.1155/2012/243524

Review Article

Molecular Biologic Approach to the Diagnosis of Pancreatic Carcinoma Using Specimens Obtained by EUS-Guided Fine Needle Aspiration

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Received 30 May 2012; Revised 13 August 2012; Accepted 15 October 2012

Academic Editor: Grazyna Rydzewska

We review the utility of endoscopic ultrasound-guided fine needle aspiration (EUS-FNA), a rapid, safe, cost-effective, and accurate diagnostic modality for evaluating pancreatic tumors. EUS-FNA is currently used for the diagnosis and staging of pancreatic tumors. The sensitivity of EUS-FNA for pancreatic malignancy ranges from 75% to 94%, and its specificity approaches 100% in most studies. However, EUS-FNA has some limitations in the diagnosis of well-differentiated or early-stage cancers. Recent evidence suggests that molecular biological analysis using specimens obtained by EUS-FNA improves diagnostic sensitivity and specificity, especially in borderline cytological cases. It was also reported that additional information regarding patient response to chemotherapy, surgical resectability, time to metastasis, and overall survival was acquired from the genetic analysis of specimens obtained by EUS-FNA. Other studies have revealed that the analysis of KRAS, MUC, p53, p16, S100P, SMAD4, and microRNAs is helpful in making the diagnosis of pancreatic carcinoma. In this paper, we describe the present state of genetic diagnostic techniques for use with EUS-FNA samples in pancreatic diseases. We also discuss the role of molecular biological analyses for the diagnosis of pancreatic carcinoma.

1. Introduction

Pancreatic cancer is now the fifth-leading cause of cancer-related death in Japan, and the annual mortality due to pancreatic cancer is estimated to be over 20,000 individuals. The 5-year survival rate of pancreatic cancer is as low as 5.5%, and the poor prognosis is attributed to the difficulty in detection of the disease at an early stage due to its high malignancy potential, the propensity of the cancer to metastasize, and the cancer's high resistance level to antitumor agents.

Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) was introduced into clinical practice in the early 1990s, and it is now considered one of the most useful methods for histological diagnosis and staging of pancreatic cancers [1, 2]. EUS-FNA of the pancreas is an efficient and minimally invasive procedure for the diagnosis and staging of pancreatic cancer. Various studies conducted since 2003 have found that EUS-FNA for pancreatic solid masses showed the following values: sensitivity, 78%–95%; specificity, 75%–100%; positive predictive value, 98%–100%; negative predictive value, 46%–80%; accuracy, 78%–95% [3]. However, specimens obtained by EUS-FNA are tiny and fragmented so that a definitive diagnosis is frequently challenging for pathologists. Nevertheless, it is important to determine the histological subtype of the tumor, especially in an unresectable tumor because the choice of treatment largely depends on the subtype [1]. It is thus, necessary to improve the diagnostic sensitivity and specificity of EUS-FNA. In this paper, we describe the current state of the genetic diagnosis techniques, and we discuss the role of molecular biological analyses for the diagnosis of pancreatic carcinoma.

2. Oncogene

2.1. KRAS (Kirsten Rat Sarcoma-2 Virus). The KRAS oncogene is frequently mutated in human malignancies such

as colon, lung, and ovarian cancer. In pancreatic cancer, mutations in KRAS are found in more than 90% of patient samples. The most frequent mutation is the constitutively active $KRAS^{G12D}$ allele. Interestingly, KRAS mutations are frequently detected in the most common precursor lesion to pancreatic cancer, pancreatic intraepithelial neoplasia (PanIN), indicating a potential role for early pancreatic cancer in the disease [4, 5].

Several research groups have suggested that the presence of KRAS gene mutations in tissue obtained by EUS-FNA improved the accuracy of the diagnosis of pancreatic cancer [3, 6–8]. Wang et al. [9] evaluated the usefulness of a novel method including EUS-FNA for the detection of mutations in the KRAS gene for the diagnosis of pancreatic cancer. They evaluated biopsies histopathologically and cytopathologically. In the pancreatic cancer cases studied, 88.9% (48/54; 95% confidence interval [CI]: 80.5%–97.2%) had KRAS gene mutations (codons 12 and 13), whereas 61.1% (33/54; 95% CI: 48.1%–74.1%) were unequivocally diagnosed by histocytopathology. Those authors also reported that compared with the measurement of serum CA19-9, the sensitivity of detection by KRAS mutations (76.2%) and the sensitivity of detection by the combination of KRAS mutations and serum CA19-9 (81%) were significantly higher than that for serum CA19-9 alone (52.4%). A logistic regression model showed that the KRAS mutation was significant (odds ratio = 5.830; CI: 1.531–22.199, $P = 0.01$), but not serum CA19-9.

Bournet et al. reported that codon-12 KRAS point mutation was found in 66% of pancreatic adenocarcinoma samples obtained by EUS-FNA [10]. No case of chronic pancreatitis displayed KRAS mutation. In that study, the following values were obtained for cytopathology alone for the diagnosis of pancreatic adenocarcinoma versus chronic pancreatitis: sensitivity, 83%; specificity, 100%; positive predictive value, 100%; negative predictive value, 56%; overall accuracy, 86%. When the KRAS mutation analysis was combined with cytopathology, these values reached 88%, 100%, 100%, 63%, and 90%, respectively. Bournet et al. also noted that the KRAS analysis in addition to EUS-FNA biopsy was useful in strongly suggesting a benign lesion, when chronic pancreatitis presented as a pseudotumor a negative finding (wild-type KRAS). Reicher et al. demonstrated that combining routine cytology with fluorescence in situ hybridization (FISH) and KRAS analyses improves the diagnostic yield from EUS-FNA of solid pancreatic masses [11].

Takahashi et al. reported that EUS-FNA with the addition of KRAS mutation analysis to the cytopathologic and histopathologic analysis was highly accurate for the differentiation of benign versus malignant pancreatic mass lesions [7]. In the 62 pancreatic cancer cases examined in the present study, with respect to cytopathologic diagnosis, the KRAS point mutation was found in 50% (2/4) of the cases with a result of no malignancy, in 71% (5/7) of the cases in which malignancy was suspected, and in 76% (39/51) of the cases in which malignancy was diagnosed (Table 1). With respect to histopathologic diagnosis (Table 2), the KRAS point mutation was detected in 43% (3/7) of the cases with a result of insufficient material, in 64% (7/11) of those with no malignancy, in 80% (4/5) of the cases with a finding of atypia, in 80% (12/15) of the cases in which malignancy was suspected, and in 83% (20/24) of the cases in which malignancy was diagnosed. These studies indicate that samples obtained by EUS-FNA analyses of KRAS mutation improve the diagnosis of pancreatic cancer. In addition, such an analysis is more useful than combining conventional methods.

Table 1: Relationship between cytopathologic evaluation and KRAS point mutation in specimens of pancreatic cancer (62 cases) obtained by EUS-FXA; citation from [7].

	Cytology	KRAS point mutation positive
No malignancy	4	2 (50%)
Suspicion of malignancy	7	5 (71%)
Malignancy	51	39 (76%)
Total	62	46

Table 2: Relationship between histopathologic evaluation and KRAS point mutation in specimens of pancreatic cancer (62 cases) obtained by EUS-FXA; citation from [7].

	Histology	KRAS point mutation positive
Insufficient material	7	3 (43%)
No malignancy	11	7 (64%)
Atypical	5	4 (80%)
Suspicion of malignancy	15	12 (80%)
Malignancy material	24	20 (83%)
Total	62	46

3. Tumor Suppressor Genes

3.1. p53. Inactivation of the p53 tumor suppressor gene is very common in almost all human cancers [12]. Normal p53 protein functions in cell-cycle regulation, in the maintenance of genomic stability, and in controlled cell death (apoptosis). A mutated p53 protein is capable of inactivating the normal function of p53 in cells, even in the presence of the normal (wild-type) protein. Most inactivating mutations in p53 consist of single point mutations in evolutionarily conserved domains that change the amino acid composition of the resulting p53 protein. The majority of inactivating mutations in p53 leads to an increased stability of the p53 protein. Under normal conditions, p53 protein levels in the cell nucleus are not detectable by standard protein immunohistochemistry, but in cells with mutated p53, the accumulation of p53 protein is easily detectable. Inactivation of the p53 tumor suppressor gene is common in pancreatic carcinoma and is found in 50%–70% of cases [13–15].

Itoi et al. conducted a p53 immunohistochemical analysis in FNA biopsy specimens obtained from chronic pancreatitis and pancreatic cancers [16]. 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

Table 3: Accuracy of the 3 tests for diagnosing pancreatic cancer; citation from [16].

Diagnosis	Test	Case (n)	Sensitivity (%)	Specificity (%)	Accuracy (%)
Pancreatic cancer	Cytology analysis	56	65	93.8	73.2
	Cytology analysis + MUC1(+)	56	85	100	89.3
	Cytology analysis + MUC5AC(+)	56	90	93.8	91.1

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% specificity, and 79% accuracy.

Jahng et al. 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 [17].

3.2. p16 (CDKN2A, INK4). The gene encoding the cell-cycle regulatory protein p16 is localized on chromosome band 9p21. Mutations in the p16 gene are associated with an increased risk of a wide range of cancers, and alterations of the gene are frequently seen in cancer cell lines. Pancreatic adenocarcinoma is often associated with mutations in the p16 gene [18, 19].

p16 mutations in EUS-FNA specimens revealed sensitivity and specificity of 13% and 100%, respectively, for a pancreatic cancer diagnosis. However, when detection by monitoring the loss of heterozygosity (LOH) was used, the sensitivity was improved to 85% for allelic losses at 9p [20].

3.3. SMAD4 (DPC4). SMAD4 is often found mutated in many cancers. It acts as a tumor suppressor that functions in the regulation of the TGF-β signal transduction pathway, which negatively regulates the growth of epithelial cells and the extracellular matrix (ECM). SMAD4 is inactivated in approximately 55% of pancreatic cancers, either by homozygous deletion (30%) or by intragenic mutations and loss of second allele (25%) [21].

LOH on 18q with SMAD4 is detected in 43% of EUS-FNA specimens from chronic pancreatitis and in 78% of EUS-FNA specimens from pancreatic cancer [20]. In the present study, using the LOH test for pancreatic cancer diagnosis at chromosomal position 18q with SMAD4, the sensitivity and specificity of the pancreatic cancer were 78% and 57%, respectively.

4. Glycosylated Proteins

4.1. MUCs. Mucins (MUCs) are heavily glycosylated high molecular weight glycoproteins with an aberrant expression profile in various malignancies [22]. It is reported that distinct gene MUC genes have been identified at least 14 genes. Under normal circumstances, mucins are known to play a protective role for epithelial tissues. Alterations in the expression and in the structure of mucins have been reported in both preneoplastic and neoplastic lesions [23, 24]. In tissue specimens of pancreatic cancer, overexpression of MUC1 (membrane-bound pan-epithelial mucin) and MUC6 (gastric pyloric gland-type secretory mucin) and the de novo expression of MUC5AC (gastric surface secretory-type mucin) have been observed as early events in pancreatic carcinogenesis in all stages of PanIN and invasive ductal adenocarcinomas, whereas goblet cell metaplasia with associated MUC2 (intestinal-type secretory mucin) expression was an extremely rare event in most of the studies.

Giorgadze et al. examined the epithelial expression profiles of MUC1, −2, −5AC, and −6 on cell block sections of EUS-FNA samples [25]. They observed the expression of MUC1 and −6 but not that of MUC2 or −5AC in nonneoplastic pancreatic samples. MUC5AC expression in differentiating ductal adenocarcinomas from benign conditions demonstrated better operating characteristics than either MUC1 or MUC6. Those authors used a panel of three antibodies, and the combination of MUC1+/MUC2−/MUC5AC+ was noted in 70.0% of the ductal carcinoma samples.

Wang et al. immunohistochemically investigated the expression of mucins (MUC1, MUC2, and MUC5AC) in EUS-FNA samples of pancreatic occupying lesions [26]: the prevalence of MUC1, MUC2, and MUC5AC expression in pancreatic cancers were 77.5% (31/40), 10.0% (4/40), and 80.0% (32/40), respectively, and in the benign pancreatic diseases the corresponding values were 25% (4/16), 31.3% (5/16), and 43.8% (7/16). As shown in Table 3, they investigated whether the combination of MUC1+cytology and MUC5AC+cytology could provide higher sensitivity and accuracy in a pancreatic cancer diagnosis in comparison with only a cytologic diagnosis. Carrara et al. found that the prevalence of MUC7 in ductal adenocarcinoma was 73.0% [27]; MUC7 expression was highly significant for adenocarcinoma ($P = 0.007$) and borderline for intraductal papillary mucinous neoplasm (IPMN) ($P = 0.05$). MUC7 was expressed in 37.5% of the chronic pancreatitis cases examined.

The MUC expression profile in EUS-FNA biopsy specimens has high value for the diagnosis of pancreatic cancer and mucinous neoplasms. It can play an important role in the clinical diagnosis of pancreatic occupying lesions.

5. Calcium Binding Protein

5.1. S100P. A member of the S100 family of calcium-binding proteins. S100P is a 95-amino-acid protein [28]. S100P expression is described in many different cancers, and its expression is associated with drug resistance, metastasis, and poor clinical outcome. S100P has been shown to mediate tumor growth, drug resistance, and metastasis through

RAGE (receptor for advanced glycation end products) [29]. Several studies have documented that S100P is highly overexpressed in pancreatic ductal adenocarcinoma [30–33]. Several studies examined the usefulness of S100P in the diagnosis of pancreatic adenocarcinoma using EUS-FNA [34–36]. Deng et al. [36] indicated that S100P has 100% sensitivity and 92.8% specificity and a diagnostic accuracy of 100% in six atypical and suspicious cases histologically proven to be pancreatic adenocarcinoma. Daniel et al. reported that S100P had 90% sensitivity and 67% specificity or diagnosing pancreatic adenocarcinoma in cytological specimens obtained by EUS-FNA. They also studied other proteins overexpressed in pancreatic adenocarcinoma (i.e., prostate stem cell antigen, fascin, 14-3-3 sigma, and mesothelin), and they confirmed that S100P was the best marker to diagnose pancreatic adenocarcinoma. These results suggest that the use of S100P in the molecular diagnosis of pancreatic adenocarcinoma using EUS-FNA can increase the diagnostic accuracy for pancreatic cancer.

6. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are small, noncoding RNA molecules of 17 to 27 nucleotides in length. miRNAs play critical roles in diverse biological processes such as cell development and differentiation and the control of cellular proliferation. MiRNAs are aberrantly expressed in virtually all human cancer types, and it has been reported that miRNAs may function as tumor suppressors or oncogenes, and that alteration in miRNA expression may play a critical role in tumorigenesis and cancer progression. In our recent study, we showed that various miRNAs are changed in gastric cancer cells by application of the antidiabetic drug metformin [37]. Preis et al. used an optical intensity analysis to investigate miRNA expression in cytokeratin 19 (CK19)-positive epithelial cells in surgically resected pancreatic cancer tissues and EUS-FNA samples [38]. In their study, the expression levels of miR-10b were increased in the pancreatic cancer cells in the EUS-FNA samples compared to the levels in pancreatic ductal cells in benign lesions. They also found that lower levels of miR-10b in the cancer cells were associated with improved response to neoadjuvant gemcitabine-based chemoradiotherapy, surgical resectability, time to metastasis, and overall survival.

However, miRNAs are a relatively new focus of molecular biological analyses in pancreatic cancer using EUS-FNA specimens. miRNAs may eventually be useful factors in the diagnosis of pancreatic cancer, but further studies are needed.

7. Molecular Diagnosis by EUS-FNA

Jones et al. reported a comprehensive genetic analysis of 24 pancreatic cancers in 2008 [39]. They found that pancreatic cancers contain an average of 63 genetic alterations, the majority of which are point mutations. These alterations defined a core set of 12 cellular signaling pathways and processes (including KRAS, TGF-β signaling and p16) that were each genetically altered in 67% to 100% of the tumors. These findings are useful for the development of not only treatments for pancreatic carcinoma, but also diagnostic approaches to this cancer. With the above described genes, the diagnosis rate for pancreatic cancer using EUS-FNA may be improved.

Many genes are reported to be related to pancreatic cancer, but there are few reports about the genes used in the molecular diagnosis by EUS-FNA. The GNAS gene encodes the α-subunit of the stimulatory G-protein (Gαs), which mediates the regulation of adenylate cyclase activity through G-protein-coupled receptors. Activating mutations of GNAS are reportedly prevalent in IPMN [40]. GNAS is mutated in approximately 60% of IPMNs and in some invasive pancreatic cancers arising in association with an IPMN [41].

BRAF is a member of the Raf kinase family of serine/threonine-specific protein kinases. This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion. The BRAF gene is activated by oncogenic RAS, leading to cooperative effects in cells responding to growth factor signals. Schönleben et al. evaluated mutations of BRAF in 36 IPMN/IPMC (intraductal papillary mucinous carcinoma) samples by direct genomic sequencing [42]. Exons 5, 11, and 15 for BRAF were examined. One missense mutation (2.7%) was identified within exon 15 of BRAF. The mutations appear to be somatic, since the same alterations were not detected in the corresponding normal tissues. Schönleben et al. argued that oncogenic properties of BRAF contribute to the tumorigenesis of IPMN/IPMC. The diagnosis of pancreatic cancer by EUS-FNA using these genes merits further studies.

8. Conclusions

EUS-FNA is a rapid, safe, cost-effective and accurate diagnostic modality for evaluating pancreatic tumors. The sensitivity of EUS-FNA for pancreatic malignancy ranges from 75% to 94%, and its specificity approaches 100% in most studies [35, 43, 44]. However, EUS-FNA has some limitations in distinguishing between well-differentiated adenocarcinoma and reactive changes, because of overlapping cytological features between neoplastic and reactive ductal epithelium. Therefore, false-negative rates and atypical or suspicious diagnoses remain relatively high.

Based on this paper, a diagnostic algorithm reflecting the most efficient approach to distinguish pancreatic cancer from benign lesions in EUS-FNA samples can be constructed. Since EUS-FNA still has the highest diagnostic relevance, reaching 100% predictive values while showing acceptable sensitivity and specificity, it should remain a preferred method for the examination of a focal pancreatic mass. Only a subset of EUS-FNA inconclusive samples should be further examined by genetic analyses, and the positive cases from molecular diagnoses using the various markers should proceed to treatment. Future studies of EUS-FNA and genetic analyses can be expected to obtain additional information toward improving response to chemotherapy,

Table 4: Studies of molecular biologic diagnosis of pancreatic carcinoma using specimens obtained by EUS-FNA.

Biological analyses	Author	Year	EUS-FNA samples	Sensitivity	Specificity	Positive predictive value	Negative positive value	Accuracy	Diagnosis
KRAS	Reicher et al. [11]	2011	46	87.9%	93.8%	96.7%	78.9%	89.8%	Combination with cytology and FISH
	Bournet et al. [10]	2009	178	88.0%	100%	100%	63.0%	90.0%	Combination with cytopathology
	Maluf-Filho et al. [8]	2007	74	90.9%	47.6%	89.5%	98.1%	89.2%	Combination with cytopathology
P53	Jahng et al. [17]	2010	61	51.0%	100%				Combination with cytology
	Itoi et al. [16]	2005	62	90.0%	91.0%			92%	Combination with cytopathology
P16	Salek et al. [20]	2007	101	85.0%	100.0%				Monitoring the loss of heterozygosity
SMAD4	Salek et al. [20]	2007	101	78.0%	57.0%				Monitoring the loss of heterozygosity
MUCs	Wang et al. [26]	2007	40	100%				91.1%	MUC5AC+cytology
	Giorgadze et al. [25]	2006	30	70%	100%	100%	100%	75%	Combination of MUC1+/ MUC2−/ MUC5AC+
S100P	Kosarac et al. [34]	2011	14	78.2%	87.5%				
	Daniel et al. [35]	2011	62	90.0%	67.0%				

*FISH: fluorescense in situ hybridization.

surgical resectability, time to metastasis, and overall survival in pancreatic cancer cases [45–47].

As shown in Table 4, summarized reports of diagnosis of pancreatic cancer by genetic analyses, our paper indicates that genetic diagnosis could be useful to improve the diagnostic sensitivity and specificity of EUS-FNA, especially in those borderline cases that cannot be rendered with certainty by morphology alone.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 781765, 10 pages
doi:10.1155/2012/781765

Review Article

Desmoplasia in Pancreatic Cancer. Can We Fight It?

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Received 25 May 2012; Accepted 17 September 2012

Academic Editor: Davor Stimac

The hallmark of pancreatic tumours, the desmoplastic reaction, provides a unique microenvironment that affects pancreatic tumour behaviour, its ability to grow and metastasize as well as resist the effects of chemotherapy. Complex molecular interactions and pathways give rise to the desmoplastic reaction. Breakdown or penetration of the desmoplastic reaction may hold the key to overcoming the limits of delivery of efficacious chemotherapy or the development of new targeted treatments. Herein we discuss such new developments to fight the desmoplastic reaction, including inhibitors of the epidermal growth factor, fibroblast growth factor, the hedgehog pathway, as well as new molecular targets like CD40 agonist and its effects on T cells, extracellular matrix modifying enzymes such as LOXL2 inhibitor and novel tumour penetrating peptides for delivery of drugs.

1. Introduction

It is well recognised that the growth of dense, collagen-rich, extracellular matrix and stroma with high interstitial pressure around pancreatic tumours, known as the desmoplastic reaction, creates a unique microenvironment that paradoxically promotes both tumour growth and metastatic spread and at the same time forms a barrier to chemotherapy penetration. Targeting components of the tumour stroma that contribute to the desmoplastic reaction is a promising new platform of investigation. Most strategies comprise of increasingly newly identified peptides that aim to enhance chemotherapeutic and even radiotherapeutic efficacy, by increasing tumour accumulation, penetration, and drug-distribution and targeting signalling pathways, which are directly implicated in the formation of desmoplastic reaction.

The hallmark of the desmoplastic reaction in tumours originating from solid epithelial glands is a dense amount of interstitial fibrillar collagen (type I and III) and accelerated proliferation of fibroblasts. Tumour-stromal interactions between pancreatic cancer cells and stromal fibroblasts lead to enhanced key gene expression promoting primary tumour incidence, tumour growth, metastasis, and angiogenesis. The tumour cells themselves are able to produce extracellular matrix (ECM) proteins and integrins [1, 2] and interact with ECM by expressing functionally active ingredients [3, 4]. The stromal production is facilitated by an abundance of growth factors including fibroblast growth factors, epidermal growth factors receptor ligands, transforming growth factor beta isoforms, and connective tissue growth factors [5]. This environment nourishes the cancer cells and facilitates invasive and metastatic potential. In this regard, any agents that target profibrotic growth factors such as small molecule tyrosine kinase inhibitors that interfere with the epidermal growth factor (EGF) receptor, FDG, platelet-derived growth factor (PDGF) receptor signalling may be useful in suppressing the proliferation of fibroblast and stellate cells (Table 1).

2. Discussion

2.1. Transforming Growth Factor Beta (TGFβ). Many growth factors expressed by human pancreatic carcinoma cells have the ability to induce fibroblast proliferation, for example, transforming growth factor β1 (TGFβ1) and fibroblast growth factor (FGF) 2 and are associated with advanced tumour stage and decreased survival.

TGFβ is a potent cytokine that regulates mammalian development, differentiation, and homeostasis and normally exerts anticancer activities by prohibiting cell proliferation,

Table 1: Classification of antidesmoplastic agents.

Agent	Class
PD 98059	MEK 1 inhibitor
U0126	MEK inhibitor
LY294002	ERK inhibitor
PP1-PP2	TβR inhibitors
SB431542 and SB525334	TβRI selective inhibitor
LY2109761	TβRI/II dual inhibitor
SD-208	TβRI inhibitor
AP 12009	TGFβ2 mRNA phosphorothioate antisense oligodeoxynucleotide
2G8	Neutralising antibody to TβR2 neutralising antibody
IPI-926	SMO Semisynthetic cyclopamine analogue inhibitor
GDC-0449	2-arylpyridine class SMO inhibitor
iRGD	Disulfide-based cyclic RGD tumour-penetrating peptide
CP870,893	IgG2 antibody to CD40
AB0023	Allosteric inhibitor of LOX-L2

motility, invasion, and metastases. In the process of tumourigenesis genetic and epigenetic events and aberrant alterations within the tumour confer TGFβ oncogenic activities, causing direct metastatic progression via stimulation of epithelial-mesenchymal transition (EMT). EMT also confers stem cell like properties to transitioned cells such as self renewal, tumour initiating capability, and chemoresistance [6].

TGFβ exerts its effects through TGFβ 1 and 2 receptors (TβR1 and TβR2), and Smad transcription regulators. TGFβ binding to TβR2 initiates a cascade that leads to Smad 2 and 3 activation, which in turn binds to Smad 4; the activated complex is transcriptionally active in the nucleus [7]. The growth inhibitory effect of TGFβ is thought to be mediated by Smad-dependent TGFβ signalling. In pancreatic defects in Smad proteins, especially Smad 4 or TβR2 lead to resistance to the growth inhibitory effects of TGFβ. These events in combination with activated K-Ras result in rapid tumour development. In human pancreatic cancer cells, TGFβ1, overexpression correlates with collagen I levels, suggesting that TGFβ1 is directly able to elicit the desmoplastic reaction, an observation which has been confirmed in experimental models of pancreatic cancer [8]. There is also cross-talk between collagen, TGFβ1, and MT1-MMP. MT1-MMP overexpression has been linked with fibrosis and various signalling pathways including Snail pathway, cadherins, Ras/MEK/ERK.

TGFβ also induces Snail family of transcription factors through the Smad pathway. In PDAC, collagen activates TGFβ signalling, in turn leading to increased Snail expression; whereas blocking TGF signalling with a highly specific TβRI inhibitor blocks collagen-induced Snail expression [9]. In addition, knocking down Smad 3 abrogates Snail-induced collagen fibrosis. Therefore TGFβ is a critical signalling pathway in the development and propagation of the desmoplastic reaction. The TGFβ pathway has been targeted using various strategies including small molecule inhibitors of TβRI, TGFβ-specific neutralizing antibodies, and antisense compounds [10].

As already discussed above, TGF binding to TβR2 receptor leads to activation of Smad proteins which mediate gene expression related to cell growth control. Part of this effect is mediated by the Ras/MEK/ERK signalling cascade. MEK 1 inhibitor PD 98059 reduced TGFβ1 related increase of tumour cell scattering migration and invasion [11358848] and enhances efficacy of gemcitabine. More recently, another molecule, Lefty, was identified downstream of the Ras/MEK/ERK pathway to mediate growth inhibition in pancreatic cell lines. Activation of the pathway in pancreatic cancer suppresses Lefty activation and enables cancer cells to escape growth inhibition. Inhibition of the pathway enhances TGF-mediated lefty upregulation with potential therapeutic applications [11]. The Smad pathway is also blocked by PP1 and PP2, Src family kinase inhibitors that inhibit TGFβ-Smad signalling [12].

TβR1 inhibitors have also been used in combination with gemcitabine in an attempt to improve chemopenetration. Two such molecules, SB431542 and SB525334 are able to augment the cytotoxic effects of gemcitabine [13]; SB525334 also increased apoptotic cell death and affected both the AKT pathway, and TβR1 receptor, the former crucial in gemcitabine resistance and the latter known to affect cell migration. In a similar fashion, LY2109761 suppressed both basal and TGFβ1-induced cell migration and invasion. In combination with gemcitabine, it reduced tumour burden, prolonged survival, and reduced spontaneous abdominal metastases [14]. The first human Phase I study of oral TβR1 inhibitor LY2157299 in patients with treatment-refractory malignant glioma is currently underway with promising results [15].

Another small molecule, SD-208, blocking TβR1, resulted in inhibition of expression of genes associated with tumour progression and inhibition of invasiveness in a cell-based assay. SD-208 treatment reduced proliferation and induced apoptosis in the primary tumours, and reduced fibrosis in the tumour microenvironment [16]. Similarly, Trabedersen (AP 12009) is a phosphorothioate antisense oligodeoxynucleotide specific for human TGFβ2 mRNA with

antitumour activity in human pancreatic cancer, such as reduction in tumour growth, lymph node metastases, and angiogenesis [17]. The TβR2 has also been targeted by specific neutralising antibodies. 2G8 an anti-rat monoclonal antibody specifically binds and blocks TβR2, inhibiting Smad 2. As a result, reducing tumour cell migration and inhibition of tumour cell migration as well as reduced EMT transcription factors are observed, which may translate in possible delayed tumour progression. This antibody has also been shown to inhibit tumour metastases in vivo [18].

More recently, further TβR molecular pathways have been identified such as the regulation of cell adhesive properties by decreasing expression of E cadherin. These results in increased expression of invasion associated integrins and integrin binding proteins, promoting invasion and metastasis, ECM and related protein production (collagen, fibronectin, decreases collagenase, heparinize, and stromelysins) as well as plasminogen activator inhibitor 1 and tissue inhibitor of metalloprotease that inhibit ECM degradation and increase proteolytic activity of cells [19]. Furthermore, there have been reports of significant association between plasma TGFβ1 and overall survival in patients with locally advanced metastatic disease, Smad 4 loss correlation with lower survival with potential important implications in treatment decision [20, 21]. Clearly the increasing understanding of TGFβ and its functions has brought a new era in molecular therapeutics. However, acquired resistance to small molecule inhibitors is a problem that has already manifested, with resultant carcinomas more aggressive and inflammatory [22]. The recent discovery that there is transcriptional talk between TGFβ and stem cell pathways holds more promising research to come [23].

3. Fibroblast Growth Factor (FGF)

Another important function of TGFβ is that it increases production of mitogenic growth factors including fibroblast growth factor. Fibroblasts are responsible for synthesis, degradation, and remodelling of ECM and can modulate behaviour of cancer cells through cytokine secretion and modification of ECM environment. Fibroblasts are thought to be mesenchymal cells, known as stellate cells, which have differentiated into myofibroblasts that secrete collagen I, which is highly resistant to proteolysis. Stellate cells are thought to mediate the invasive potential of PDAC cells and promote EMT [24] as well as resistance to radiotherapy [25]. FGF mediates its effects through different receptor isoforms. In particular, FGFR1 IIIb isoform is associated with inhibition of cancer cell proliferation, migration, and invasion, whereas FGFR1 IIIc enhances cell proliferation. FGFR2 IIIb increases venous invasion but FGFR2 IIIc is associated with metastases, more aggressive tumours and confers PDAC cells features suggestive of cancer stem cells [26]. The FGF binding protein is dramatically upregulated in pancreatic cancer and is linked to the initiation and progression of pancreatic cancer [27]. Various preclinical studies have shown FGFR signalling inhibition may play a role in inhibiting tumour growth [28]. Neutralising monoclonal antibodies to FGF2 has been shown to suppress hepatocellular cancer growth by blocking angiogenesis and inhibiting downstream cellular signalling.

4. CD44 and Hyaluronan

Another key role of fibroblasts in the desmoplastic reaction is hyaluronan synthesis and its interaction with CD44. CD44 is another integral cell-surface glycoprotein; overexpression of its variant forms, driven by IFN gamma, has been associated with malignant transformation of pancreatic tumours [29, 30]. In fact, pretreatment levels of CD44 and its variants have been correlated with TNM staging and may well be able to serve as tumour markers in head and neck cancers [31].

CD44 is also critical in pancreatic carcinogenesis as it is the major cell surface receptor for hyaluronan, as well as matrix metalloproteinases. Hyaluronan, is a glycosaminoglycan, able to interact with extracellular matrix molecules (hyaladherins) affecting matrix structure but also cell function through its interaction with CD44, making it another key component of the stromal reaction. In addition, its breakdown products, via hyaluronidase activity, promote angiogenesis and in turn tumour neovascularisation [32]. Hyaluronan is produced by fibroblasts in response to factors released from tumour cells, such as lactate, or by direct cell-cell contact [33]. Hyaluronan-rich stroma is associated with poor prognosis in many epithelial cancers including pancreatic and together with CD44 promotes tumour cell growth, migration, and metastases [33, 34]. It is thought that hyaluronan provides increased barrier integrity and chemoresistance through CD44-dependent reorganisation of the tumour cytoskeleton [35], where as the anti-CD44 monoclonal antibody IM7 (anti-CD44 IgG2b mAb IM7) improves vascular permeability [36]. Disruption of the hyaluronan-CD44 interaction is a key therapeutic target to prevent tumour refractoriness secondary to drug resistance [37]. One such strategy implores a hyaluronan synthesis inhibitor, 4-Methylumbelliferone (4-MU), has been shown to inhibit cell migration, proliferation, and invasion [38, 39]. The ability of 4-MU to suppress hyaluronan synthesis and accumulation has recently been linked to suppression of bone metastases in breast cancer [40]. Its inhibitory effect has been shown to slow down the development of human pancreatic cancer cell lines in vitro and in mice [41, 42] but also to enhance the efficacy of gemcitabine [43]. In a similar fashion, the action of PEGylated human recombinant PH20 hyaluronidase (PEGPH20) acting as a hyaluronan depletor improved chemopermeability of doxorubicin and gemcitabine and when given in combination with the latter led to inhibition of pancreatic tumour growth and improved survival over gemcitabine alone (median survival 28.5 days versus 15) [44, 45].

5. Hedgehog Pathway

Hedgehog is a signalling pathway that is genetically altered and aberrantly activated in the majority of pancreatic cancers leading to tumour initiation, progression, and metastatic spread. In addition, it has been implicated in the initiation and maintenance of the desmoplastic reaction (Figure 1).

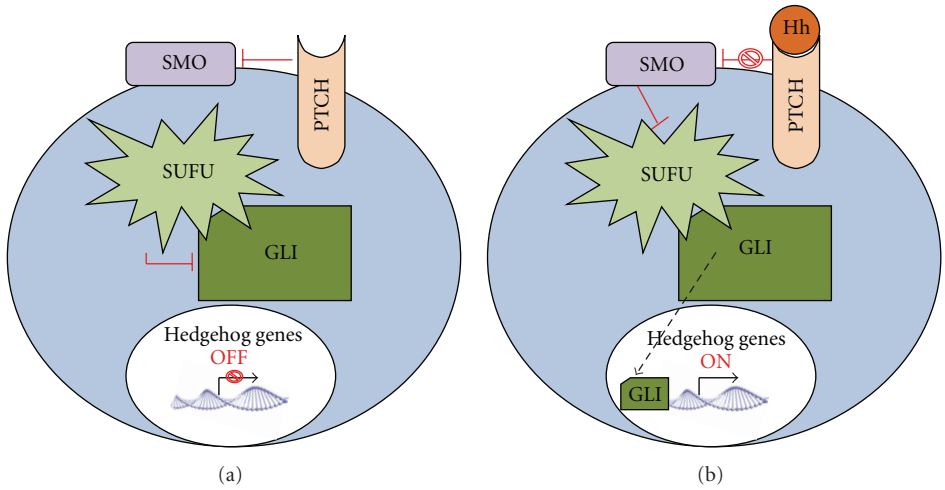

Figure 1: The Hedgehog pathway [46].

The hallmark of the desmoplastic reaction is a dense amount of interstitial fibrillar collagen (type I and III) and accelerated proliferation of fibroblasts. The latter are thought to be mesenchymal cells, known as stellate cells, which have differentiated into myofibroblasts that secrete collagen I, which is highly resistant to proteolysis. Hedgehog (HH) signalling promotes myofibroblast differentiation and induces stroma-derived growth promoting molecules, which are in turn tumourigenic. In addition, HH ligands induce matrix metalloproteinases and TGFβ1, which are both highly active in the desmoplastic reaction formation and directly involved in fibrosis. The pathway is activated when sonic hedgehog ligands (SHH) bind to the patched receptor (PTCH) relieving the inhibitory effects of Patch (PTCH) on smoothened (SMO) and activating the GL1 family of transcription factors which turn on the Hedgehog genes such as PTCH, epidermal-derived, platelet-derived, and vascular-endothelial growth factors, cyclins B, D, and E and GLI1. Bulk cancer cells secrete hedgehog ligands to activate the pathway in stroma and cancer stem cells, promoting the formation of desmoplastic reaction and facilitating maintenance of cancer stem cells involved in metastases. Ectopic production of HH ligands has been associated with pancreatic tumourigenesis [47]. In addition, overexpression of SMO in cancer-associated stromal fibroblasts has been observed that in turn activates the HH signalling pathway [48]. Evidence also suggests that tumour cells secrete HH ligand to induce tumour-promoting HH target genes in a paracrine fashion in adjacent stroma to support tumour growth [49, 50].

Blocking the hedgehog pathway in vitro studies, with the small molecule cyclopamine, a naturally occurring antagonist of the hedgehog signalling pathway component (smoothened-transmembrane receptor), leads to abrogation of pancreatic metastases and potential improvement in chemodelivery [51, 52]. IPI-926 a semisynthetic cyclopamine analogue was developed to inhibit SMO. It has been shown to reduce the desmoplastic reaction and increase tumour vascular density by blocking hedgehog signalling and hence blocking metastatic spread and tumour initiation. Inhibition of Hedgehog signalling has been shown to enhance the delivery of drugs in vitro [53] and can occur in many platforms including HH ligand inhibition, SMO antagonism, and Gli transcriptional activity inhibition.

Several studies have been designed to assess the synergistic function of Hedgehog inhibitors delivered alongside with established antineoplastic agents [54]. In one such study, Stephenson et al. tested the safety profile of IPI-926 in previously untreated metastatic pancreatic cancer in a phase Ib trial. They noted that IPI-926 facilitated the delivery of gemcitabine by diminishing tumour-associated desmoplasia with 31% of patients showing partial response and 63% showing reduction in CA 19-9. Treatment was confounded by grade 3 toxicity fatigue and transaminitis. A randomised double-blind placebo-controlled study is underway to assess survival comparison between the treatment and placebo arms, where the treatment arm will receive daily 160 mg oral IPI-926 plus gemcitabine infusion at 100 mg/m^2 once weekly for 3 weeks of a 28-day cycle [NCT01130142]. Unfortunately the Phase II trial by Infinity was recently stopped because of futility of treatment [55].

Another SMO inhibitor, GDC-0449/Erivedge, also known as vismodegib, is an orally administrable molecule 2-arylpyridine class that inhibits SMO and is highly selective for SHH-Gli signalling, though to act by inhibiting SHH pathway at the level of Gli genes. Gli signalling has been implicated in the regulation of cell proliferation, cell cycle, and cell survival. GDC-0449 has been shown to inhibit pancreatic cancer cell viability, Gli-DNA binding and transcriptional activity and induces apoptosis in three pancreatic cancer cell lines and stem cells [56]. It also inhibited expression of HH receptors, such as Patched and SMO and effectors. Preclinical studies have demonstrated antitumour activity in xenograft models of pancreatic cancer [57]. LoRusso et al. presented their Phase I trial results in 2011 utilising GDC-0449 in patients with refractory, locally advanced or metastatic solid tumours, including 8 with pancreatic cancer [58] [21300762]. The molecule was able

to produce tumour responses in 20 patients with BCC and medulloblastoma. The best observed response for pancreatic cancer was seen in one patient with stable disease at 2.8 months. Most promising was that Gli1 downregulation was noted and the treatment was associated with low toxicity. Recently following Phase II trials in BCC, the drug was approved by the FDA for the treatment of metastatic or locally advanced BCC that cannot be treated with surgery or radiotherapy. The trial showed partial response in 30% of patients with metastatic disease and complete or partial response in 43% of patients with locally advanced disease (ERIVANCE trial BCC/SHH4476g AACR). The theory behind GDC-0449 altering HH signalling is being tested in a Phase II study with vismodegib in the preoperative setting for patients with local, resectable disease to detect change in HH signalling in the normal tumour surrounding tissue (Proof of Mechanism Study of an Oral Hedgehog Inhibitor GDC-0449 in Patients With Resectable Pancreatic Ductal Adenocarcinoma in the Pre-operative Window Period, also known as HIPPoS by Cambridge University Hospitals NHS Foundation, NCT01096732, estimated primary completion date September 2012) looking at whether blocking the HH pathway will directly affect tumour cells or the surrounding normal tissue.

One of the main reasons for ultimate resistance to therapy is due to the existence of cancer stem cells which are resistant to chemotherapy and lead to treatment failure. The Michigan group are currently evaluating the combination of vismodegib with gemcitabine for patients with advanced disease and its effect to cancer stem cells and HH pathway (cancer stem cells and inhibition of HH pathway signalling in advanced pancreas cancer: a pilot study of GDC in combination with gemcitabine-NCT01195415), in a hope that pretreatment with GDC-0449 will inhibit the HH pathway in cancer cells and downstream tumour microenvironment enhancing treatment efficacy for gemcitabine. One of the primary endpoints is to evaluate the effect of HH signalling inhibition on pancreatic cancer stem cells by assessing the number of cancer stem cells before and after GDC-0449 treatment. Preliminary results of this trial show that three out of five patients who received pretreatment with GDC-0449 followed by gemcitabine treatment showed partial response, reduction in CA 19-9 levels, and increased vacuolated structures in tumour cells of one patient. The estimated primary completion date for this study is June 2013.

With a similar target in mind, another open label, single arm, multicentre Phase II trial is currently evaluating the progression free survival in patients with metastatic adenocarcinoma treated with vismodegib in combination with gemcitabine and nab-Paclitaxel (a Phase II Study of Gemcitabine and Nab-Paclitaxel in Combination With GDC-0449 (Hedgehog Inhibitor) in Patients With Previously Untreated Metastatic Adenocarcinoma of the Pancreas by Sidney Kimmel comprehensive Cancer Centre at John Hopkins-NCT01088815, estimated primary completion date December 2012). Abraxane is thought to weaken the stroma allowing for better chemotherapeutic efficacy of gemcitabine, using GDC-0449 to destroy the stroma but also to kill cancer stem cells. Furthermore Abraxane has shown clinical activity in patients overexpressing secreted protein acidic and rich in cysteine (SPARC), as it binds to the albumin portion of paclitaxel, potentially providing a tool to reverse gemcitabine resistance. Measurement of SPARC levels may also serve as a prognostic factor for treatment success [59, 60].

Other Phase I trials currently underway are assessing combination treatments with GDC-0449 such as in combination with Sirolimus or Erlotinib and Gemcitabine. Preliminary results are encouraging and have shown disease stabilisation and low drug-related toxicities (DLTs) for Erlotinib with Gemcitabine and GDC-0449. (Gemcitabine Hydrochloride With or Without GDC-0449 in Treating Patients With Recurrent or Metastatic Pancreatic Cancer by University of Chicago NCT01064622 to assess progression free survival; Sirolimus and Vismodegib in Treating Patients With Solid Tumours or Pancreatic Cancer That is Metastatic or Cannot Be Removed By Surgery by Mayo Clinic NCT01537107, primary completion date January 2014; GDC-0449 and Erlotinib Hydrochloride With or Without Gemcitabine Hydrochloride in Treating Patients With Metastatic Pancreatic Cancer or Solid Tumours That Cannot Be Removed by Surgery by Mayo clinic NCT00878163). Preliminary results are showing stable disease and low DLTs [61].

An important consideration is that SMO is localised in the primary cilium of the cell, which is critical in HH signalling and cancer progression. Primary cilia are required for the activation of the HH pathway in normal cells but are lost in many cancers. Some drugs may be ineffective in the absence of primary cilia [62]. Hence further research into overcoming this barrier should be considered when designing new platforms.

6. iRGD: a Tumour Penetrating Peptide for Peptide-Mediated Delivery of Drugs

One of the main reasons for treatment failure remains inability to penetrate the stromal reaction and the generation of elevated intratumour interstitial pressure. Crossing the vascular wall and penetrating into the tumour parenchyma is the main challenge for efficacious drug delivery. Recent attention has been paid to penetrating peptides for peptide-mediated drug delivery, especially peptides containing an RGD integrin recognition motif which allows them to bind to av integrins on the tumour cell surface. However to date, conventional RGD peptides have only been able to penetrate blood vessels but not the extravascular tumour parenchyma. A newly devised peptide, iRGD, a disulfide-based cyclic RGD peptide, seems to have overcome this obstacle by also targeting a downstream receptor, neuropilin-1. iRGD is a synthetic peptide containing a motif that binds to av integrins on tumour endothelium. Upon binding, the peptide is proteolytically cleaved to expose a CRGDK fragment, losing its integrin affinity but gaining affinity for neuropilin-1 instead. The new complex triggers tissue penetration, thus this peptide penetrates through the tumour vasculature into the tumour parenchyma [63].

Since the peptide is able to penetrate into the tumour parenchyma, coupling of the peptide with drugs may

improve the drug delivery and efficacy, especially as iRGD seems to home to tumours but not normal tissue. av integrin and neuropilin-1 expression is largely restricted to tumours but most importantly the response is tumour specific because the peptide cleavage will only occur if there has been prior integrin activation. The hypothesis has been tested in mouse tumour models including pancreatic adenocarcinoma where various drugs including doxorubicin, nab-paclitaxel (abraxane), and doxorubicin liposomes as well as trastuzumab were coadministered with the peptide, without the need for chemical conjugation therefore preserving drug activity and improving tolerability. Tumour accumulation was increased 12-fold for abraxane, 14-fold for the doxorubicin liposomal nanoparticle, and 7-fold for the free drug and 40-fold for trastuzumab indicating that iRGD leads to enhance drug delivery to cancer cells [64]. The manufacturing company has already initiated SBIR trials with iRGD in combination with gemcitabine with preliminary data showing that iRGD enhances the anti-tumoural activity of gemcitabine in orthotopic models of pancreatic cancer [65].

7. CD40 Agonist

CD40 is a type I transmembrane glycoprotein receptor of the TNF-receptor superfamily widely expressed by immune cells such as dendritic cells, B cells, and macrophages but also endothelial cells, smooth muscle cells, fibroblasts, and epithelial cells. The CD40 ligand (CD40L) primarily expressed in the surface of activated T cells interacts with CD40+ B cells to produce multiple regulatory signals including T-cell and B-cell-dependent proliferation, immunoglobulin production and switching, and apoptosis. CD40L+T cells augment the antigen-presenting function of CD40+ B cells and other antigen-presenting cells (APCs) generating a number of interactions between CD4 and CD8 T cells [66, 67].

Interestingly, CD40 is also expressed in the membrane and cytoplasm of tumour cells but is absent from non-proliferating tissues. Its activation promotes apoptotic death and generation of tumour specific T-cell responses that contribute to tumour elimination [68]. The exact mechanism of CD40-CD40L interaction is still unclear as CD40 expression has been correlated with worse tumour prognosis, TNM stage, and lymph node metastases, perhaps because the CD40L is rarely expressed on pancreatic cancer TILs and hence unable to downregulate CD40+ cancer growth. In fact, presence of CD40L expression has been linked to improved survival [69]. In addition, epigenetic alterations of miRNA-regulated CD40 expression lead to downregulation of CD40 expression in pancreatic cancer cells promoting invasion and metastasis [70]. CD40 also engages in endothelial cells to induce in vitro tubule formation and expression of matrix metalloproteinases [71]. In a recent Phase I trial by He et al. [72], recombinant soluble human CD40L was used to block CD40 and demonstrated significant growth inhibitory effect in vitro. Specifically they showed the ligand was able to cause not only growth arrest but also cancer cell apoptosis. CD40 binding antibodies have the potential to modulate pancreatic cancer cell growth. Binding of recombinant soluble CD40L or with a CD40 reactive monoclonal antibody may produce a direct inhibitory effect on cancer cells. CD40 agonist antibody CP-870,893 can achieve substantial regression of tumours in some patients with inoperable pancreatic binding antibodies may bind to epitopes distinct from those involved in the natural CD40-CD40L interaction. Similarly CD40 monoclonal antibodies may cause collateral activation of antibody dependent cellular cytotoxicity.

CP-870,893 is a fully human IgG2 antibody that selectively interacts with CD40 at a distinct site from its ligand-binding region. Binding enhances MHCII expression as well as dendritic cell activity and is therapeutically effective against several CD40 + human tumours. In a Phase 1 dose escalation open label study CP-870,893 was combined with gemcitabine in patients with chemotherapy naive surgically incurable pancreatic cancer [73], tumour regression was observed a subsequent mouse model that tumour regression was T cell and gemcitabine independent but dependent on macrophages, that infiltrated the tumour and facilitated the depletion of the tumour stroma. Soon underway a small open label single-arm Phase I study looking at preoperative gemcitabine together with CP870,893 followed by addition of CP-870,893 to adjuvant chemoradiotherapy for patients with newly diagnosed resectable pancreatic cancer. Patients will receive standard surgery followed by chemoradiotherapy; one dose of gemcitabine/CP870,893 will be preoperatively and 3 doses postoperatively.

8. LOX-L2

Lysyl oxidase like 2 belongs to the lysyl oxidase family of extracellular matrix modifying enzymes. This group of enzymes plays an important role in connective tissue biogenesis, cellular adhesion, motility and migration, gene transcription regulation, and senescence, as well as cancer progression. Increased LOX-L2 expression has been identified in many cancers including the pancreas. In breast cancer, high levels of LOX-L2 expression appear to correlate with decreased overall survival and metastases free survival ($P = 0.023$ and $P = 0.0367$, resp.) [74]. Interestingly, LOX-L2 does not appear to be required for primary tumour growth but enables metastases in vivo.

LOXL2 serves as an extracellular matrix metalloenzyme and has been shown to catalyse the first step in the formation of crosslinks in fibrillar collagen and elastin [75, 76]. Cross-linking of collagen activates other enzymes involved in matrix remodelling such as MMPs, enhancing tumour cell invasion [77]. Therefore LOX-L2 is directly able to modify the ECM, and its overexpression leads to propagation of the desmoplastic reaction. Positive association between LOX-L2, TIMP1, and MMP9 has also been noted in human colorectal cancer [78–80]. LOXL2 inhibition has also been associated with reduction in activated fibroblasts, endothelial cells, desmoplasia, and decrease in transforming growth factor-beta signalling making LOX-L2 a potential target for fighting the desmoplastic reaction [81].

Preclinical evidence suggests that in vivo blocking LOXL2 both in vivo and in vitro is highly effective in preventing

distant metastases in breast cancer through regulation of tissue inhibitor of metalloproteinase 1 (TIMP1), leading to increased TIMP1 and MMP 9 activity and facilitating ECM remodelling [82].

In pancreatic cancer cell lines, gene silencing by inhibition with small interfering RNAs has been shown to result not only in cell death but also in increased sensitivity to gemcitabine treatment [83]. In this study, LOXL2 appeared to regulate E2F5 transcription factor associated with invasion and metastases. Blocking not only LOXL2 but its effectors too, such as E2F5 or even RAMP3, a molecule downstream of LOXL2 thought to mediate some of its tumourigenic activity [81], might also prove beneficial as antitumourigenic agents.

In addition, development of specific allosteric inhibitors of LOXL2, such as AB0023, bind remote to its catalytic domain, allowing inhibition of LOXL2 regardless of substrate concentration [84]. This concept has many prospects: the ability to confer a molecule high specificity and selectivity for the cancer without affecting normal tissues, development of high affinity binders, and using different specificities of LOXL2 targeting antibodies to alter the outcome.

More excitingly, recently an intracellular function of LOXL2 has been described for the first time in relation to E-cadherin and histone H3; In normal cells, methylation of lysine 4 within histone 3 activates CDH1 transcription and E-cadherin formation, while histone deacetylation plays an important role in downregulation of E-cadherin in human pancreatic cancer promoting tumour cell migration and proliferation [85]. Loss of the cell adhesion molecule E-cadherin is critical in pancreatic tumourigenesis. LOXL2 has been found to act in the nucleus of cancer cells and deaminates the lysine 4 amino group of H3 leading to downregulation of CDH1, decreased E-cadherin expression, fewer cellular adhesions facilitating tumour growth and metastases [86].

9. Radiotherapy

As already mentioned above, there is data suggesting that pancreatic stellate cells confer protection against radiotherapy through β1-integrin and FAK signaling [25]. β1-integrin signaling and in particular integrin-mediated adhesion to extracellular matrix proteins has been implicated in mediating cell survival in response to radiation in different cancer cell lines [87]. Other PSC-specific matrix proteins such as periostin, stimulate growth, and confer resistance even under the effects of radiotherapy, continuing to enhance the desmoplastic reaction by producing excessive extracellyular matrix proteins [88]. Inhibition of the pathway enhances the efficacy of radiotherapy [30, 89]. More recently the role of caveolin-1 (Cav-1) as a critical signaling molecule within the β1-integrin and FAK pathway was described. Knockdown models of caveolin-1 increased radiosensitisation in human pancreatic cell lines [90]. Further research in this domain is required to enhance in vivo radiosensitivity.

10. Conclusion

Increasing understanding of the desmoplastic reaction and the heterogeneity of alterations of signalling pathways in pancreatic cancer is already providing us with new insights into how to fight desmoplasia. Preliminary evidence encourages the idea that attenuating the desmoplastic reaction may help limit the molecular and clinical course of pancreatic cancer, contain its progression, and enhance the response to chemotherapy. There is a long way to go until this evidence will become practice.

Conflict of Interests

The authors have no potential conflict of interests.

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Hindawi Publishing Corporation
Gastroenterology Research and Practice
Volume 2012, Article ID 473960, 5 pages
doi:10.1155/2012/473960

Clinical Study

Is Post-ERCP Pancreatitis a Genetically Predisposed Complication?

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Received 16 February 2012; Revised 19 June 2012; Accepted 9 July 2012

Academic Editor: Grazyna Rydzewska

Background/Objectives. Pancreatitis remains the most common complication of ERCP. History of post-ERCP pancreatitis is an independent risk factor for a new episode, suggesting a genetic background. The N34S mutation in serine protease inhibitor Kazal type 1 (SPINK 1) gene may downregulate the threshold for the development of pancreatitis. The aim of the present study is to evaluate the presence of this mutation among patients with post-ERCP pancreatitis. *Methods.* During a period of four years, thirty patients with post-ERCP pancreatitis entered the study. Patients and procedural data were collected, focusing on risk factors for pancreatitis. Blood samples were taken for genetic testing for the presence of N34S mutation in SPINK 1 gene. After DNA extraction, we used an allele-specific polymerase chain reaction as an initial screening method for the N34S mutation, and in order to confirm the results and to determine the hetero- and homozygosity genotype status, we used a restriction fragment length polymorphism (RFLP) method. *Results.* None of the thirty patients was found to carry the N34S mutation, with both of the applied methods. Patients had an average of two of the known risk factors. *Conclusion.* SPINK1 N34S mutation does not seem to play a role in post-ERCP pancreatitis, but larger studies needed to confirm our results.

1. Introduction

Pancreatitis remains the most common complication of ERCP, with the reported incidence ranging from 2% to 9% [1]. Although 80% of cases are mild, a significant number of patients may develop severe pancreatitis, that means additional morbidity and risk for death. ERCP, despite the development of new diagnostic tools, remains a widely used procedure, so post-ERCP pancreatitis is a problem with significant impact.

So far only the use of pancreatic stents in high risk patients has become a widely accepted practice to minimize the risk for post-ERCP pancreatitis [2]. However, this technique is costly and not widely available, so the question who are high risk patients remains vivid.

Several studies and meta-analyses helped us to recognize special factors that put an individual in high risk for the development of post-ERCP pancreatitis [3–10]. Among these factors special interest presents the history of post-ERCP pancreatitis as an independent risk factor for a new episode of post-ERCP pancreatitis. It seems that some individuals have a genetically predisposed susceptibility in this particular complication.

Genetic factors, such mutations in genes of cationic trypsinogen and CFTR, are known to play a causal role in the development of certain types of chronic pancreatitis [11].

Table 1: Sequences of oligonucleotide Primers of the SPINKI gene.

Primers	Allele-specific PCR	RFLP method
Sense	5′-CAATCACAGTTATTCCCCAGAG-3′	5′-TTCTGTTTAATTCCATTTTTAGGCCAAATGCTGCA-3′
Antisense	5′-GTTTGCTTTTCTCGGGGTGAG-3′	5′-GGCTTTTATCATACAAGTGACTTCT-3′
Mutation	5′-CCATTTTTAGGCCAAATGTTACAG-3′	

PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism.

Mutation in another gene, that codes the serine protease inhibitor Kazal type 1 (SPINK 1), has been found to act complementary to other genetic or environmental factors causing pancreatitis [12–14].

The aim of the present study is to investigate the possible role of SPINK 1 gene mutations in the development of post-ERCP pancreatitis, examining the incidence of these mutations in this particular group of patients in comparison with the general population.

2. Patients and Methods

For this purpose, a study was conducted in two high volume centers (more than 200 ERCPs/Y each). Between the years 2005 and 2008, the data of each "ERCP case" were collected according to a standard protocol. Patient data including demographics, past history and the indication for the procedure, were collected before the ERCP. Procedural data, including difficulty in cannulation (number of attempts on papilla), use of precut, pancreatic duct catheterization and opacification, findings of the examination, sphincterotomy, and all the other therapeutic maneuvers were recorded at the time of the procedure. All patients were followed up at least for 24 hours to monitor the development of complications after ERCP. Patients that developed post-ERCP pancreatitis, according to the widely accepted criteria (new onset of pancreatic-type abdominal pain, lasting at least for 24 hours, associated with a 3-fold increase in serum amylase in the same period) entered the study, after an inform consent was obtained. Exclusion criteria from the study were the presence of chronic pancreatitis and the refusal of consent. From the patients that entered, the study blood samples were collected for genetic analysis of SPINK1 gene. Patients were monitored during their hospitalization, and severity of pancreatitis was classified according to the consensus criteria [15].

3. Mutation Analysis

Genomic DNA was extracted from peripheral blood leucocytes according to established protocols using the Whole Blood DNA isolation Kit (Qiagen GmbH, Germany). Concentration of DNA solutions were determined by UV-Vis spectrophotometer. In order to identify N34S SPINK1 mutation as a possible risk factor for the post-ERCP pancreatitis, we applied the following approach. We used an allele-specific polymerase chain reaction as an initial screening method for the N34S mutation, according to a previously described report [16]. In order to confirm the results and to determine the hetero- and homozygosity genotype status, we used a restriction fragment length polymorphism (RFLP) method as previously described, with slight modifications [17].

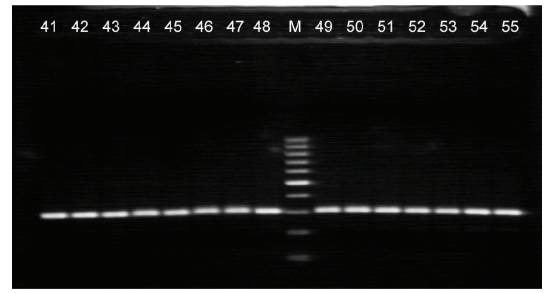

Figure 1: Allele-specific PCR analysis of the N34S mutation on exon 3 of the SPINK gene. The wild-type SPINK band is located at 285 bp. If the N34S mutation is present, a second band at 190 bp appears. The lanes 41 to 55 represent patients without the N34S mutation (normal). The lane marked with M represents the 100 bp DNA Ladder (Fermentas Life Sciences, Lithuania).

3.1. Allele-Specific PCR. Briefly, 200 ng of genomic DNA were subtended to a 50 μL reaction, containing 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 2 mM $MgCl_2$, 250 μM dNTPs, 3.0 U Taq DNA polymerase (Bioron International, Germany), and 100 ng of sense, antisense and mutation primers (see Table 1). The following cycling conditions were used for the above PCR reaction: an initial denaturation step at 94°C for five minutes, then 35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and a final elongation step at 72°C for five minutes. PCR products were electrophorized on a 2% agarose gel and stained with ethidium bromide for UV visualization. This allele-specific PCR generates two fragments (285 bp and 195 bp) when the N34S mutation is present, and one fragment of 285 bp for the wild-type genotype. Figure 1 provide an overview of the results obtained with the allele-specific PCR.

3.2. RFLP Analysis. The primers we used for the RFLP analysis were designed (by Threadgold et al.) to amplify exon 3 of the SPINK1 gene, based on the published nucleotide sequence (GenBank, NM-003122). These primers (see Table 1) introduce a PstI endonuclease restriction site in sequences carrying the N34S variant, and a BsrDI endonuclease restriction site in wild-type sequences. Polymerase chain reaction was performed in a 50 μL reaction volume, using 100 ng of genomic DNA template, 0.5 U Pfu DNA polymerase (Promega, Germany), 2 mM $MgCl_2$, 200 μM dNTPs (Fermentas Life Sciences, Lithuania), and 10 pmol of

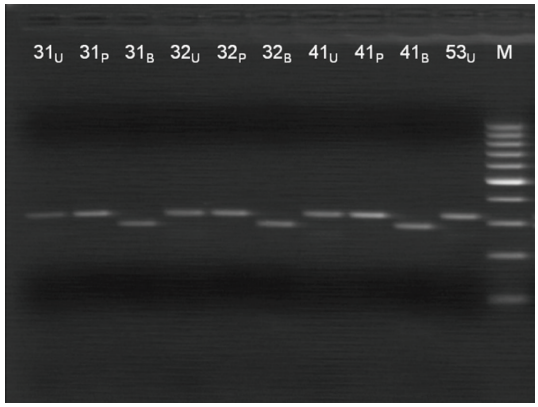

Figure 2: An overview of results obtained with N34S Restriction fragment length polymorphism analysis. Samples 31, 32, 41, and 53 are wild types. $_U$undigested PCR product, $_P$CR product digested with *Pst*I, $_B$CR product digested with *Bsr*DI.

each primer. The cycling conditions we used for the RFLP method were as follows: an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Then, the PCR products were digested with restriction endonucleases PstI and BsrDI. Twenty microliters of the PCR product was added to 10 U PstI (Fermentas Life Sciences, Lithuania), 1x digest buffer (Fermentas Life Sciences, Lithuania), and 1x bovine serum albumin (BSA) (Fermentas Life Sciences, Lithuania) to a final volume of 50 μL and incubated at 37°C for one hour. The BsrDI digestion was performed in a 50 μL reaction, consisting of 20 μL PCR product, 10 U BsrDI (Fermentas Life Sciences, Lithuania), 1x digest buffer (Fermentas Life Sciences, Lithuania) and 1x bovine serum albumin (BSA) (Fermentas Life Sciences, Lithuania) and incubation at 55°C for one hour. Heat inactivation of the digestion reaction was performed for both digestions at 80°C for 15 minutes. The digestion products analysed by agarose gel electrophoresis using a 3% (w/v) precast agarose gels (Biorad Laboratories Inc., USA), 1X TAE buffer, and 0.5 mg/ml ethidium bromide. The undigested PCR products were 0320 bp in length. After digestion with PstI restriction endonuclease, a product of 286 bp obtained from mutant sequences. After digestion with BsrDI, a product of 286 bp obtained from wild-type sequences. Heterozygote samples produced both products of 320 bp and 286 bp after digestion with either endonuclease. To validate the RFLP analysis further, the BsrDI digestions was performed alternatively at 37°C overnight. The results obtained were identical in both experiments. An overview of results obtained with the RFLP methods are provided by Figure 2.

4. Results

Between the years 2005 and 2008, 1162 patients underwent 1247 procedures, mainly for therapeutic purpose. Pancreatitis developed in 34 patients (2,7%). From these, 30 patients finally entered the study. From the remaining 4, one had chronic pancreatitis, and three refuted consent. From the 30 patients who entered the study, 21(70%) were women and 9 were men (30%), with a mean age of 63 years old (STDEV 13). Pancreatitis was classified, according to Cotton criteria [15], as mild in 17 patients (57%), moderate in 10 patients (33%), and severe in 3 patients (10%). We did not detect the N34S mutation among our study group with both methods of, allele-specific PCR and RLFP. Patients had an average of 2 risk factors/per pt. None of the patients in our group fulfilled the criteria of SOD, and we did not have patients with prior history of post-ERCP pancreatitis. The distribution of the remaining risk factors is presented in Table 2. The mean number of guidewire passes into the pancreatic duct was 2,47 (STDEV 3,46).

Table 2: Risk factors for the development of post-ERCP pancreatitis.

Risk factors	Number of patients	Percentages (%)
Age <60 y	11	37
Female gender	21	70
History of pancreatitis	3	10
Difficult cannulation ≥5 attempts on papilla	5	17
Precut	3	10
Pancreatogram	18	60

5. Discussion

SPINK 1 is a 6,5 KDa protein consisting of 79 amino acids, including a 23 amino acid signal peptide. It is synthesized in the pancreatic acinar cell and its central role is the protection of the pancreas from the prematurely activated trypsin. It has been estimated that SPINK 1 is capable of inhibiting up to 20% of trypsin within the pancreas, but SPINK/trypsin ratio likely varies depending on the state of inflammation in the gland. SPINK 1 protein is coded from a gene on the chromosome 5, that is approximately 7.5 Kb long, and contains four exons. The N34S mutation, caused by an c.101A > G transition in exon 3, was firstly reported by Chen et al. in 2000 [12]. However, the association between N34S mutation and pancreatitis became evident in two subsequent studies. Initially Witt et al. found a strong association of this mutation with "idiopathic" chronic pancreatitis, and they further speculated that these mutations cause the disease in an autosomal recessive manner [13]. However, Pfützer et al. in another major study suggested that these mutations, which are also present in about 2% of the healthy population, do not cause the disease by themselves, but rather modify the disease, possibly by lowering the threshold for pancreatitis from other genetic or environmental factors [14]. Several studies worldwide have confirmed the association between SPINK 1 mutations and idiopathic chronic pancreatitis, and as a consequence other groups investigate the role of these mutations in other types of chronic pancreatitis. So SPINK 1 mutations were found to be associated with tropical pancreatitis and to a lesser degree with alcoholic chronic pancreatitis [18–25]. Moreover, a recent study revealed that

these mutations may play a role in recurrent episodes of acute pancreatitis [26].

Post-ERCP pancreatitis is a type of acute pancreatitis that shares the same pathophysiological characteristics with any other type of acute pancreatitis. As we know, history of post-ERCP pancreatitis puts an individual in high risk for the development of a new episode of post-ERCP pancreatitis, independently from the other known factors. This implies that some individuals may have a genetic susceptibility in this complication. The N34S mutation, as mentioned earlier, may act complementary to another factor such as the irritation of the gland during ERCP for the development of pancreatitis. Lempinen et al. found that patients with post-ERCP pancreatitis had significantly higher levels of SPINK 1 from patients without pancreatitis, as a consequence of the induction of enzyme synthesis and secretion by the inflammation [27]. SPINK1 gene mutations may downregulate enzyme's protective role against the irritation that is caused to the gland by the ERCP maneuvers.

Despite this logical approach, our study did not reveal this particular mutation among patients with post-ERCP pancreatitis. In contrast, patients had an average of two of the known risk factors. The limitation of the study is the small size, due to lack of many cases of post-ERCP pancreatitis. Moreover, data about the incidence of SPINK1 mutations in our country are lacking and maybe our ethnic origin represents a population with a low burden of these mutations. However, the fact that some persons are more susceptible than others, independently from the presence of other risk factors, strongly suggest the role of an unknown genetic factor. Large multicenter studies are needed to evaluate this hypothesis, examining the presence of this or other mutations in SPINK 1, or other relevant genes, in order to extract safer results.

Conclusively according to our data, we have not got any clues that N34S SPINK 1 mutation plays a role in post-ERCP-pancreatitis, but further studies are needed to confirm our results.

Conflict of Interests

The author declare that there is no conflict of interests.

Acknowledgments

The author would like to thank for their support to this study Professor of Immunology George Kyriazis, his colleagues Mr. Hosam Abu Uda and Mrs. Olga Christopolou, and finally Professor George Tatsios.

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