University of Medicine and Pharmacy Craiova

Doctoral School

PhD Thesis

Role of Genetic Polymorphisms in Pancreatic Pathology

Abstract

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GENERAL PART

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GENERAL PART

INTRODUCTION

Pancreatic pathology is widely encountered, constantly increasing with variable incidence from one continent to another, depending on ethnicity, genetic predisposition and dietary habits. Pancreatic pathology includes: congenital abnormalities, acute and chronic pancreatitis, pancreatic exocrine and endocrine tumors, genetic diseases of the pancreas, hereditary pancreatitis. From all pancreatic pathology we will focus on acute and chronic pancreatitis, pancreatic tumors, pathology with a higher prevalence and incidence in clinical practice. This thesis aims to research the correlation between genetic polymorphisms and pancreatic pathology studied (acute pancreatitis, chronic pancreatitis and pancreatic cancer) using genetic analysis.

Chapter 2

2.1 Epidemiology data

Regarding the incidence of acute pancreatitis there are considerable geographical differences between different states, for example, in the Netherlands [4] and the UK [5] there is a low incidence (10-24 patients / 100,000 inhabitants / year), but Scandinavian countries [6-8] and the USA [9] have a higher incidence (35-73 patients / 100,000 inhabitants / year) [2]. Also there are regional differences in terms of the precipitant factor and etiology of acute pancreatitis. For example, in Finland [8] and the USA [10] the leading cause of acute pancreatitis is alcohol, while studies in Hong Kong [11], England [5], Italy and Greece [12] showed more frequently the biliary cause for pancreatitis [2].

Chronic pancreatitis has a variable and uneven geographic distribution according to the onset of major risk factors involved, namely alcoholism and malnutrition, where the incidence is increasing. The incidence of chronic pancreatitis varies geographically, from 1.6 new cases / 100,000 inhabitants / year in Switzerland, up to 23 new cases / 100,000 inhabitants / year in Finland [16]. In 70% of all cases of chronic pancreatitis the etiology was alcohol [16].

Malignant pancreatic tumors are represented in approximately 90-95% of cases by pancreatic adenocarcinoma, originating in the ductal cells [21]. Pancreatic ductal adenocarcinoma is recognized as one of the most aggressive forms of neoplasia and currently it is the 5th leading cause of death from cancer after gastric, breast, lung and prostate cancer. [22,23]. Statistical data show overall survival of approximately 0.2-2% [25]. In Europe,

pancreatic cancer is the 10th in frequency among cases of cancer, accounting for approximately 2.6% of cases in both sexes, and the eighth leading cause of death from malignancy with approximately 65,000 deaths each year [26]. As geographical distribution, the highest incidence has been described in North America and New Zealand with over 30,000 new cases per year, an intermediate incidence has been described for Europe and Japan and the lowest incidence in Africa and India. In many European countries, the mortality rate of pancreatic cancer is increasing for both sexes. The mortality rate is currently at 8-12 / 100,000 / year for males and 4.6 / 100,000 / year for females [27].

2.2 Causes and associations

Etiologic factors involved in acute pancreatitis are multiple, both pancreatic and extrapancreatic, having a predisposing or triggering role. In 80-90% of patients with acute pancreatitis, etiology is dominated by gallstone (biliary acute pancreatitis) and alcohol abuse (alcoholic acute pancreatitis) varying according to geographical zone and ethnic structure of the population [1]. Significant risk factors for pancreatic cancer include smoking and non-0 blood groups [17]. Red meat, especially when prepared at high temperatures increases the risk for pancreatic cancer [37]. Obesity considerably increases the severity of acute pancreatitis [17] and is a risk factor for many cancers, including pancreatic cancer. Acute pancreatitis induced by drugs is primarily an idiosyncratic reaction, many drugs are cited as having a role in causing acute pancreatitis [17]. No drugs are known to cause chronic pancreatitis. Although less frequently, hypertriglyceridemia can cause chronic pancreatitis [60]. Celiac disease increases the risk of pancreatitis about 3 times (hazard ratio 2.85) [62]. The risk of acute and chronic pancreatitis is also elevated in patients with inflammatory bowel disease, systemic lupus erythematosus, although accurate estimates are not available [17]. Low incidence of pancreatic cancer in patients with chronic pancreatitis (<5%), demonstrating that chronic pancreatitis is a rare cause of pancreatic cancer [17].

2.3 Molecular biology and tumor progression

Numerous studies estimated that about 5-10% of malignant pancreatic tumors have a genetic cause. Major hereditary syndrome associated with pancreatic cancer are represented by hereditary non-polyposis colon cancer, familial breast cancer associated with gene mutations BCRA2, FAMM or p16, ataxia-telangiectasia, and von Hippel-Lindau disease. Cystic fibrosis and hereditary chronic pancreatitis have an increased risk of pancreatic cancer, known that the

risk of developing pancreatic cancer is about 50 times higher in patients with hereditary chronic pancreatitis than the general population. The most common modifications (approximate frequency is indicated in parentheses) include mutations of the K-ras oncogene (90%), p53 (85%), SMAD4 / DPC4 (50%), and p16 (85% mutant and 15% silent epigenetic) [73] which are accompanied by genomic and transcriptomic changes that facilitate the disruption of cell cycle, cell survival and promote invasion and metastasis [72]. Progression from minimum dysplastic epithelium (pancreatic intraepithelial neoplasia grade 1A and 1B) to more severe dysplasia (pancreatic intraepithelial neoplasia grade 2 and 3), and finally invasive carcinoma is parallel to the accumulation of successive mutations which include activation of KRAS2 oncogene, inactivation of the CDKN2A gene tumor suppressor (encoding cyclin dependent kinase 4 inhibitor), and finally, inactivation of tumor suppressor genes TP53 and DPC4 (SMAD4) [76]. SPINK1 and CTRC genes that are associated with chronic pancreatitis and should rather be considered as major contributing factors than causal, the only gene associated constantly with chronic pancreatitis is CFTR [88].

Chapter 3

3.1 Clinical diagnosis present and future

Acute pancreatitis begins suddenly and dramatically in most of cases with upper abdominal pain (95% of patients), radiating to the back, or in the bar (50%). Nausea and vomiting (initially food, then bilious, but never faeces) accompanying pain in 80% of cases, abdominal distention occurs in 75% of patients, abdominal defense and sensitivity (50% of cases), plus moderate fever (38 ° C), anxiety, agitation and confusion (pancreatic encephalopathy). Jaundice can be given by gallstones (biliary pancreatitis) or after compression CBP which appear in the cephalic pancreatitis. Dosage of serum and urine amylase remains the usual diagnostic investigation in emergency conditions, but due to lack of specificity and relative prognostic value, its values do not correlate with the severity of acute pancreatitis. The diagnosis of acute pancreatitis is most often determined by the presence of two of the following three criteria: abdominal pain; serum amylase and / or lipase greater than three times the upper limit of normal, and / or changes in abdominal imaging.

Chronic pancreatitis is characterized by recurrent episodes of abdominal pain associated with both endocrine and exocrine pancreatic insufficiency and progressive atrophy of the

pancreatic parenchyma [89]. In addition to pain, chronic pancreatitis can lead to pseudocysts, fistulas, upper gastrointestinal bleeding due to ruptured esophageal varices (caused by trombosis of portal or splenic vein), stenosis of biliar or duodenal duct, ascites and pancreatic cancer [89]. Classic triad: pancreatic calcifications, steatorrhea, and diabetes suggest the diagnosis, but appear together only in very advanced stages of the disease [90].

Pancreatic cancer has a clinical picture which may be one or more of the following signs and symptoms, most of which are nonspecific. The pain is deaf, aggravated by food intake with intense evolution in crisis. Mechanisms are obstructive, mechanically (by distension) and chemical. Weight loss is due to loss of appetite and exocrine pancreatic insufficiency with malabsorption. Transit disorders appear late, due to local invasion or peritoneal metastases. Severe digestive intolerance may appear through duodenal tumor obstruction. Jaundice is a mechanical type, with dark urine and pale stools; occurs early in localizations in the pancreatic head by tumor compression, and late in body or tail locations where compression is given soon by adenopathy and / or metastasis. Glucose intolerance or beginning of diabetes may be the first sign of a pancreatic tumors, possibly due to tumor secretion of a pancreatic islet amyloid polypeptide (PIAP). Biochemical changes frequently can be grouped into four syndromes: cholestasis syndrome due to obstruction, anemic syndrome that can accompany anorexia and malabsorption, exocrine pancreatic insufficiency syndrome, decrease glucose tolerance syndrome, due to PIAP secretion.

3.2 Imaging techniques – Ultrasound

Ultrasound is often the first imagistic exploration performed in practice in Romania, mainly due to accessibility and low cost, being a non-invasive method, extremely effective in patient emergency with a high clinical and biological suspicion of acute pancreatitis. Ultrasound as a method cannot identify chronic pancreatitis in the early stages, having factors that depend on the examiner and the patient, overweight, abdominal bloating which prevent visualization of the pancreas and individual variety of shape, appearance and echogenicity of the pancreas [96]. Diagnosis, staging and prognosis of pancreatic ductal adenocarcinoma cananot be performed using transabdominal ultrasound (still widely used in Romania) because of its low accuracy and its role is considered modest for early diagnosis of pancreatic cancer [100], due to low sensitivity and specificity compared with conventional CT and EUS.

Endoscopy ultrasound

EUS is so far the most promising imaging modality for the diagnosis of chronic pancreatitis [106]. EUS a is non-surgical method with the highest sensitivity in detecting pancreatic benign or malignant formations [110]. The EUS sensitivity of the pancreatic formations detection is 98%, which is superior to conventional computed tomography sensitivity of 86% when using modern computerized tomography system with 32 or 64 spira (multidetector CT). EUS is also higher than CT examination for determining preoperative the resectability of the tumor in non-metastatic pancreatic cancer [111]. Using fine needle aspiration (FNA) EUS-guided increase the specificity of method to 100% with a high sensitivity between 80% and 95% with an accuracy up to 99% [114]. EUS with FNA is considered superior to CT and MRI for the diagnosis of pancreatic neuroendocrine tumors.

Computer tomography

Computed tomography is considered the golden standard in the diagnosis of acute pancreatitis [94], assessing the changes in the pancreatic, peripancreatic region as well as to the distance, it is useful in the differential diagnosis with other abdominal diseases such as perforation associated with a gastroduodenal ulcer [124]. Computed tomography cannot exclude early stage of chronic pancreatitis, but has a higher sensitivity in the detection of pancreatic calcifications compared with ultrasound [96]. Spiral CT is considered the current modality of choice used for diagnosis and initial staging of patients with clinical and ultrasound suspicion of pancreatic cancer [125]. The sensitivity of spiral CT for the diagnosis of pancreatic cancer is about 70-85%, superior to transabdominal ultrasound and conventional CT which had sensitivity under 50-75%.

Magnetic resonance (MRI)

Magnetic resonance techniques have the advantage of assessing the pancreas, vascular and biliary structures or pancreatic duct, in a single examination, have been proposed for the initial evaluation of patients with pancreatic cancer [131]. Magnetic resonance techniques allow lesion detection and evaluation of differential diagnosis benign-malign, staging loco-regional and to distance, respectively assessing resectability.

3.3 Molecular techniques – polymorphisms

In pancreatic injury chain, several genetic factors like cytokines (IL-1; IL-1 receptor antagonist; IL-6; IL-10; TNF-α; IL-1β), angiogenesis-related factors (VEGFR-2; CXCR-2; PAR-

1; EGF; TGF-β), pattern-recognition receptor (CD14) and inducible nitric oxide synthase (iNOS) genes may play an important role, concerning severity and evolution of the inflammatory process [132]. A recent publication showed that the Ser608Leu iNOS SNP was significantly associated with the risk of developing acute pancreatitis and showed a higher frequency of TT genotype in patients with acute pancreatitis compared with healthy controls [132]. Ser608Leu iNOS (rs2297518) can also be used as a marker to assess the risk of acute pancreatitis [132]. Angiogenesis-related factors as VEGF, VEGFR-2, RET, EGF, TGF-β play an important role in outcomes of pancreatic cancer [149]. Studies of VEGF gene polymorphisms (+405G/C but not -460T/C and +936C/T) demonstrated an association with susceptibility to pancreatic adenocarcinoma [150]. Recently, it has been shown that VEGFR2-906 C>T polymorphism has a significant impact in pancreatic cancer as a predictor for survival and tumor recurrence [148,151]. Thus, VEGFR2 polymorphisms may represent an important prognostic marker for pancreatic cancer [151]. All studies conducted in the last years revealed that assessment of cytokines, angiogenesis-related factors, pattern-recognition receptor or inducible nitric oxide synthase genes polymorphisms may provide an important molecular and genetic tool that can be used to identify subgroups in the population with high risk for pancreatic disorders, establish patients' prognosis and choose optimal course of therapy.

SPECIAL PART – PERSONAL CONTRIBUTION

Chapter 4

4.1 Objective

The research theme is very topical because it explores and improves the recent discoveries in the field of pancreatic pathology (acute pancreatitis, chronic pancreatitis and pancreatic neoplasm) based especially on evaluating polymorphisms. The current study aimed to extend knowledge related to the genetic polymorphisms involved in pancreatic pathology. We considered that currently the literature contains limited studies on genetic polymorphisms involved in pancreatic pathology, and their results are subject to controversy. Biological markers have the advantage of being measurable over carcinogenesis cascade thus enabling their standardized measurement and can also be applied to early diagnosis of pancreatic tumors or to identifying high-risk patients, allowing close monitoring. In the identification of prognostic markers of survival in patients with pancreatic pathology we try to answer the question: is the

prognostic marker more accessible through a blood sample, for example, without need of tissue specimen?

4.2 Material and methods

In the study lot were included patients hospitalized in the Gastroenterology Clinic, 1st Surgical Clinic and 2nd Surgical Clinic of the Emergency County Hospital Craiova. The total number of patients included in the study with pancreatic pathology was 168. Pancreatic pathology was represented by acute pancreatitis (n = 110), chronic pancreatitis (n = 25), pancreatic cancer (n = 33). The control lot was composed by patients who do not have the pancreatic pathology (n = 232). Following the approval of the Ethics Committee of Craiova University of Medicine and Pharmacy, blood samples were collected from the patients from the two lots needed to study polymorphisms. Before sampling the biological material for the study all patients signed informed consent after being explained the details and clarified any ambiguities arisen. Identification of VEGFR2 and iNOS gene polymorphisms was performed in the Human Genomics Laboratory, of the Research Centre in Gastroenterology and Hepatology from the University of Medicine and Pharmacy Craiova. The protocol of identification genetic polymorphisms included the following steps: isolating genomic DNA from blood, spectophotometric assessment, identification of allelic variants by using Real Time PCR technique with the TaqMan probe and interpretation results.

4.3 Results

For data processing we used Microsoft Excel (Microsoft Corp., Redmond, WA, USA) with XLSTAT suite for MS Excel (Addinsoft SARL, Paris, France) and the IBM SPSS Statistics 20.0 software (IBM Corporation, Armonk, NY , USA). The information obtained was stored in Microsoft Excel file, then processed statistically to analyze the relationship between clinical and laboratory data of patients. Processing data - descriptive analysis of lot depending on various parameters, calculating fundamental statistical parameters, mean, standard deviation, their report, called the coefficient of variation, graphic representation - was performed with Excel, using Pivot Tables, Functions-Statistical, Chart and Data Analysis module. For achieving complex statistical tests (Chi square test, Student's test, ANOVA test) were used commands from XLSTAT module or were performed using SPSS. Although division of patients on age decades shows differences between the study and the control group, the average age of the two groups did not differ significantly ($p = 0.419$ Student test> 0.05), overall younger patients with acute

pancreatitis being offset by cancer patients with older age, reaching to an average similar to patients in the control group. Patients in the study group, are smokers in a greater proportion than those in the control group, the difference observed is statistically significant. There is very strong correlation (very important agreement) between the assessment of acute pancreatitis by Ranson score, Balthazar score and the Atlanta classification.

4.4 Polymorphisms results

In the study group variant GG of VEGFR2 is available in more than 25% of patients, while in the control group was found in only 16%. In conclusion, we can say that there may be an association between the presence of GG genotype of VEGFR2 and pancreatic pathology. We analyzed the prevalence of iNOS genotypes in the study and control group and we found no significant differences between the two groups. Although there are some differences in terms of distribution of the cases with different diagnoses (pancreatic cancer, acute pancreatitis and chronic pancreatitis) by iNOS genotype identified, these differences are not statistically significant. I have not noticed differences in the distribution for cases with different diagnoses (pancreatic cancer, acute pancreatitis and chronic pancreatitis) by VEGFR2 genotype identified, which was not statistically significant.

4.5 Discussion

The latest data from the literature, show that pancreatic pathology is a widely frequent pathology in the latest period, and pancreatic adenocarcinoma has the most severe digestive cancer prognosis, survival at 5 years was 5%. An early diagnosis is the only chance to extend life of these patients. According to the results from our study we observed that VEGFR-2 genotypes encountered in significantly different proportions in the two groups, and in the group with pancreatic pathology GG variant is available in more than 25% of patients, which is why we can say that there may be an association between the presence of VEGFR-2 GG genotype and pancreatic pathology studied in our group. Regarding the prevalence of iNOS genotypes, according to the results from our study we observed that there are no significant differences between the two groups.

4.6 Conclusions

We found that for the polymorphism VEGFR2 (KDR) - 604A> G, the presence of GG genotype is associated with a risk of about two times more likely to develop inflammatory pathology or localized pancreatic tumor. In the dominant model, carriers of the G allele (AG +

GG genotypes) have a risk of about 1.4 times more likely to develop acute or chronic pancreatitis or pancreatic cancer. Polymorphism results for iNOS2 - 2087> G were not statistically correlated with the risk of patients for developing inflammatory pathology or localized pancreatic tumor. Although the study group was not very large, it respected the Hardy-Weinberg statistical equilibrium. Most of the results and the conclusions of the study performed are consistent with the scientific literature.

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