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Original scientific paper

PREVALENCE OF S AND Z ALPHA 1-ANTITRYPSIN MUTATIONS IN PATIENTS WITH PANCREATIC DISEASES IN SERBIAN POPULATION

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One of the key points in research of pancreatic disease pathology is further elucidation of the role of proteases and antiproteases, since their imbalance can lead to pancreatic injury. Alpha 1-antitrypsin (AAT) is one of the most important serum inhibitors of proteolytic enzymes, including pancreatic enzymes trypsin, chymotrypsin and elastase. It is speculated that

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mutations in the *AAT* gene may influence the onset and the development of pancreatic disease. The presence of the most common AAT mutations Z and S was analyzed in 160 patients with pancreatic diseases (50 patients with pancreatic cancer, 50 patients with chronic pancreatitis and 60 patients with type 2 diabetes mellitus) and 129 healthy individuals by PCR-mediated site-directed mutagenesis (PSM) method. One patient with pancreatic cancer was found to be a carrier of Z mutation, as well as one patient with type 2 diabetes mellitus. One patient with chronic pancreatitis was found to be a carrier of S mutation. The common AAT mutations were statistically significantly over-represented in patients with pancreatic diseases (3 of 160 patients, allelic frequency 0.9%) than in the control group (1 of 129 individuals, allelic frequency 0.4%). The results of this study, requiring confirmation, suggest that common AAT mutations Z and S may be associated with a modest increase in susceptibility to the development of pancreatic disease.

Key words: alpha 1-anitripsin, pancreatic cancer, chronic pancreatitis, diabetes mellitus type 2, Z and S mutations

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INTRODUCTION

Pancreatic cancer, chronic pancreatitis and type 2 diabetes mellitus are major types of chronic pancreatic pathology, but their etiology, genetics and underlying molecular mechanisms are still poorly understood (OTSUKI and TASHIRO 2007; WATANABE *et al.* 2007). One of the key pathological events in the development of pancreatic disease appears to be imbalance between proteases and their inhibitors within the pancreatic parenchyma (WEISS *et al.* 2008). It is known that mutations in the gene encoding cationic trypsinogen (PRSS1) play causative role in the hereditary form of chronic pancreatitis (TEICH and MÖSSNER 2008). Other genes encoding proteases and their inhibitors, such as the anionic trypsinogen (PRSS2) and the serine protease inhibitor Kazal type 1 (SPINK1), have also been found to be associated with pancreatic disease.

Alpha 1-antitrypsin (AAT) is the major serine protease inhibitor present in the blood (DEMEO and SILVERMAN 2004). Its main function is protection of lower respiratory tract tissue from proteolytic damage by neutrophil elastase, but it also inhibits a variety of proteases in other tissues, including trypsin, chymotrypsin and pancreatic elastase in the pancreatic parenchyma. The AAT protein is involved in processes which could all be important for maintenance of normal pancreatic function, like regulation of reactive oxygen species toxicity, cell-mediated immunity/tolerance, neutrophil and endotoxin mediated inflammation, endothelial function and apoptosis (SANDSTROM *et al.* 2008). The role of AAT is of particular interest in chronic pancreatitis, considering that pancreatitis is most likely caused by inappropriate activation of pancreatic zymogens to active enzymes within the pancreatic parenchyma, leading to autodigestion and inflammation of the pancreas (TRUNINGER *et al.* 2001). The role of AAT also appears to be of importance in the

development of pancreatic cancer, considering that AAT protein is overexpressed in malignant pancreatic tissue (TRACHTE *et al.* 2002). Recent finding that AAT protects endocrine pancreatic cells from apoptosis have pointed out that this molecule plays an important role not only in exocrine, but also endocrine pancreatic tissue (ZHANG *et al.* 2007).

Gene encoding for AAT is highly polymorphic, with more than 120 allelic variants identified to date (DEMEO and SILVERMAN 2004; ZAIMIDOU *et al.* 2009). On the basis of plasma level and protein function, these variants are categorized as normal, deficient, null and dysfunctional. The most frequent AAT genetic variant is a glutamine to lysine substitution at codon 342 in exon 5 (E342K) giving rise to AAT protein variant PiZ (HUTCHISON 1998). The Z variant is both deficient and dysfunctional and in homozygous state can be the cause of severe AAT deficiency. The Z mutation gives only 15% of normal AAT serum levels in homozygotes and approximately 60% in heterozygotes (CROWTHER *et al.* 2004). Another common AAT genetic variant is a glutamine to valine substitution at codon 264 in exon 3 (E264V) giving rise to AAT protein variant PiS. The S variant is associated with increased risk of lung disease in combination with the Z variant (HUTCHISON 1998). Heterozygous carriers of S mutation have AAT levels 80% of the normal AAT plasma levels; whereas homozygous carriers have AAT levels that are 60% of the normal AAT plasma levels (CROWTHER *et al.* 2004). The role of AAT deficiency is mostly investigated in lung disorders, such as lung emphysema, chronic obstructive pulmonary disease and lung cancer (DEMEO and SILVERMAN 2004; LJUJIC *et al.* 2006; YANG *et al*. 2008; TOPIC *et al.* 2010).

Laboratory diagnosis of AAT deficiency is routinely performed by phenotyping methods, which include measurement of serum alpha-1-antitrypsin concentration and isoelectric focusing (IEF). Several DNA-based methods are also used for AAT deficiency testing, but they still have not become part of routine diagnostics (LJUJIC *et al.* 2008). There are a limited number of studies determining the frequency of common AAT variants by genotyping, especially for patients with pancreatic disease. To date, the role of *AAT* gene mutations in chronic pancreatitis remains unclear, while only few data are available regarding their potential role in pancreatic cancer and type 2 diabetes mellitus. The aim of this study was to estimate the prevalence of common AAT genetic variants, Z mutation and S mutation, in a series of Serbian subjects and to determine whether these mutations are associated with pancreatic diseases.

MATERIALS AND METHODS

This study has encompassed 160 patients with three major types of chronic pancreatic pathology, pancreatic cancer, chronic pancreatitis and type 2 diabetes mellitus. The patients with pancreatic cancer and chronic pancreatitis were referred to the Clinic of Gastroenterohepatology and the First Surgical Clinic of the Institute for Digestive Diseases in the period 2004-2007. The patients with type 2 diabetes mellitus were referred to the Department of Endocrinology of the University Clinical Center “Zvezdara” in the period 2003-2006.

The group of patients with pancreatic cancer consisted of 50 individuals (30 male and 20 female, 61.2±11.4 years). Diagnosis of pancreatic cancer was made based on clinical findings (abdominal ultrasound, computed tomography, nuclear magnetic resonance and endoscopic ultrasound) and laboratory findings (tumor marker CA 19-9 test). The diagnosis was confirmed by patohistological examination. The group of patients with chronic pancreatitis consisted of 50 individuals (42 male and 8 female, 52.0±12.4 years). Diagnosis of chronic pancreatitis was made based on the presence of exocrine and endocrine pancreatic insufficiency and the findings of morphological examinations: calcification in pancreas detected by ultrasound, computed tomography or nuclear magnetic resonance of abdomen, morphological changes of the pancreatic canalicular system detected by endoscopic retrograde cholangiopancreatography and morphological changes of the pancreas detected by endoscopic ultrasound.

The group of patients with type 2 diabetes mellitus consisted of 60 individuals (20 male and 40 female, 59.1±10.0 years) in whom diabetes was diagnosed using glucose cut-off values as defined by the WHO. Microvascular complications of diabetes, polyneuropathy and retinopathy, were diagnosed by neurological examination (clinical examination, EMNG, monofilament testing and Neuro-pad testing) and FOU examination.

Control group consisted of 129 healthy blood donors (93 male and 36 female, 40.1±10.5 years) who were referred to the National Blood Transfusion Institute in the period 2000-2002. They were included in the study based on standard blood analyses, blood pressure measurement and absence of chronic diseases.

Genomic DNA was extracted from peripheral blood using GFX^{TM} Genomic Blood DNA Purification Kit (Amersham Biosciences). The presence of the most common mutated AAT alleles, Z and S, was detected by PCR-mediated site-directed mutagenesis (PSM) method (DIVAC *et al.* 2004). Fragments containing Z and S mutations were coamplified in the same reaction. Primers used for detection of Z mutation were GCCGTGCATAAGGCTGTGCTGACCATCGT*C and TTGAGGAGCGAGAGGCAGTT. Primers used for the detection of S mutation were CCTGATGAGGGGAAACTACAGCACCTC*G and CAGTCCCAACATGGCTAAGAGGTG. Both forward primers introduce the recognition site for the same restriction enzyme, TaqI. The amplification was carried out in a 25μL mixture containing 1x reaction buffer (50mM KCl, 10mM Tris-HCl (pH 9), 0.1% Triton X-100), 2.5mM $MgCl_2$, 0.2mM of each dNTP, 5pmol of each primer, 1U of Taq polymerase (Promega) and 100-200ng of DNA. The amplification conditions were as follows: initial denaturation at 94°C for 5 minutes, 35 cycles of 30 second denaturation at 94°C, 30 second annealing at 60°C, 30 second extension at 72°C, with final extension of 10 minutes at 72°C. The obtained PCR product was digested with TaqI restriction enzyme (New England BioLabs) for 3h at 65°C. The digestion products were subjected to electrophoresis in 3% agarose gel in 1xTAE buffer at 100V for 2h. The bands were visualized by ethidium bromide staining. The normal alleles are cut with TaqI, giving raise to fragments of 181bp and 29bp for Z fragment and 119bp and 27bp for S fragment.

Differences in allele and genotype frequencies between the analyzed groups were tested by SPSS software (version 10.0.1 for Windows) using Fisher's exact test. The obtained p-values were adjusted for age and sex using logistic regression analysis, with $p \leq 0.05$ considered statistically significant.
Written informed consent was obtained from all study participants and the investigation was approved by the hospital's ethical committee.

RESULTS

To our knowledge, this is the first study analyzing the presence of the most common AAT variants by genotyping in patients with pancreatic cancer and type 2 diabetes mellitus. Only one previous study reported on AAT genotyping in chronic pancreatitis patients (WITT *et al.* 2002).

The results of genotyping performed in 160 patients with pancreatic disease and 129 controls are given in Table 1. The presence of Z and S mutations in the *AAT* gene was detected in 3 of 160 patients (allelic frequency 0.9%), while in control group 1 of 129 individuals was a mutation carrier (allelic frequency 0.4%). Statistical analysis showed no significant differences between the patients groups and the control group. However, statistically significant over-representation of the AAT S and Z mutations was found in all pancreatic disease cases combined in comparison to the control group (p=0.044).

Table 1. Distribution of mutated AAT alleles in patients and in control group

	chromosomes analyzed	S allele	Z allele	mutated chromosomes (allelic frequency)	
pancreatic disease	320	1	2	3	(0.9%)
pancreatic cancer	100	0	1	1	(1.0%)
chronic pancreatitis	100	1	0	1	(1.0%)
type 2 diabetes mellitus	120	0	1	1	(0.8%)
control group	258	0	1	1	(0.4%)

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In our study, the prevalence of common AAT variants in group of patients with pancreatic cancer showed no difference from the control group. So far, there have been no data regarding the prevalence of deficient AAT variants in patients with pancreatic carcinoma using genotyping. The concentration of AAT was found to be elevated in plasma of pancreatic cancer patients, but no difference was found in phenotype distribution among patients and controls (TOUNTAS *et al.* 1985). AAT protein is overexpressed in malignant pancreatic tissue (TRACHTE *et al.* 2002). The elevated levels of AAT in pancreatic cancer could be the consequence of the

inflammatory response observed to be associated with malignancies or it could represent a protective mechanism against the proteases produced by the malignant cells. The AAT deficiency could lead to the uncontrolled degradation of pancreatic tissue as a result of protease-antiprotease imbalance and to an increased inflammatory response.

The speculation of AAT involvement in chronic pancreatitis was prompted by several studies (EDMUNDS and WILKINSON 1991; RABASSA *et al.* 1995). Since AAT acts as a protease inhibitor, it could be important in prevention of pancreatic tissue autodigestion. Increased AAT concentration was found in pancreatic juice of patients with chronic pancreatitis (MISZCZUK-JAMSKA *et al.* 1983). It was speculated that this could alter the balance in pancreatic protease-antiprotease system and subsequently lead to chronic pancreatitis. Only one study, beside this one, used genotyping to determine the frequency of Z and S mutations in chronic pancreatitis patients (WITT *et al.* 2002). In both studies no statistical difference was found in distribution of Z and S mutations between patients and controls. When the studies using measurement of AAT concentration and phenotyping are taken into account, the conflicting data are obtained regarding the involvement of the AAT variants in chronic pancreatitis. Some findings indicated increased prevalence of PiS and PiZ in chronic alcoholic patients, while others indicated no difference (BRAXEL *et al.* 1982; EDMUNDS and WILKINSON 1991; RABASSA *et al.* 1995).

The idea about AAT involvement in type 2 diabetes mellitus was raised by several functional studies, which showed that AAT prevents both diabetes formation in non-obese diabetic mice and B-cell apoptosis in vitro (LU *et al.* 2006; ZHANG *et al.* 2007). To our knowledge, this is the first study investigating association of *AAT* gene variants with type 2 diabetes mellitus using genotyping. Recently, the frequency of AAT deficient variants, determined by IEF phenotyping, was shown to be increased in patients with type 2 diabetes mellitus compared to non-diabetic control subjects (SANDSTROM *et al.* 2008). Also, the number of individuals with low AAT levels (<1.0 mg/ml) was 50% higher in the diabetic group ($p<0.05$). Several other studies showed abnormal AAT levels in patients with type 1 diabetes mellitus, but no phenotyping or genotyping for these patients was done (SANDLER *et al.* 1988; LISOWSKA-MYJAK *et al.* 2006). In our study, the increase in frequency of mutated alleles in type 2 diabetes mellitus patients compared to the control group was not statistically significant. The reason for this could be a small number of patients involved in the study.

Our study showed that there is a statistically significant increase in the frequency of the tested mutated alleles in patients with pancreatic diseases in comparison to the control group, which could indicate importance of the *AAT* gene for the pathogenesis of pancreatic diseases. Confirmation of the results in larger cohort of the patients and in different populations is needed. It would be also beneficial to screen the whole *AAT* gene, considering the number of identified AAT variants (DEMEO and SILVERMAN 2004, LJUJIC *et al.* 2006). The results of this study suggest that common AAT mutations Z and S may be associated with a modest increase in susceptibility to the development of pancreatic disease.

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UČESTALOST S I Z MUTACIJA U GENU ZA ALFA 1-ANTITRIPSIN KOD PACIJENATA SA BOLESTIMA PANKREASA

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I z v o d

Jedan od osnovnih izazova u proučavanju patologije bolesti pankreasa predstavlja dalje razjašnjavanje uloge proteaza i antiproteaza, zbog toga što poremećena ravnoteža između njih može dovesti do oštećenja pankreasa. Alfa 1-antitripsin (AAT) je jedan od najvažnijih inhibitora proteolitičkih enzima u serumu, među kojima su i enzimi pankreasa: tripsin, himotripsin i elastaza. Pretpostavlja se da mutacije u *AAT* genu mogu da utiču na pojavu i razvoj bolesti pankreasa. Prisustvo najčešćih mutacija u *AAT* genu, označenih kao Z i S, analizirano je u 160 pacijenata sa bolestima pankreasa (50 pacijenata sa kancerom pankreasa, 50 pacijenata sa hroničnim pankreatitisom i 60 pacijenata sa dijabetesom tipa 2) i u 129 zdravih osoba. Prisustvo mutacija detektovano je analizom dužina restrikcionih fragmenata. Jedan pacijent sa kancerom pankreasa je bio heterozigotni nosilac Z mutacije, kao i jedan pacijent sa dijabetesom tipa 2. Jedan pacijent sa hroničnim pankreatitisom je bio heterozigotni nosilac S mutacije. Dve najčešće mutacije u *AAT* genu su bile statistički značajno učestalije kod pacijenata sa bolestima pankreasa (3 / 160 pacijenata, alelska frekvencija 0,9%) nego u kontrolnoj grupi (1 / 129 osoba, alelska frekvencija 0,4%). Rezultati ove studije, koje ukazuju na moguću povezanost Z i S mutacija sa umerenim povećanjem rizika za razvoj bolesti pankreasa.

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