GENETIC INVESTIGATIONS IN CHRONIC PANCREATITIS AND PANCREATIC CANCER

Ph.D. Thesis

Anita Balázs, M.D.

First Department of Medicine
Faculty of Medicine
University of Szeged

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Anita Balázs, M.D.

Supervisors:

Prof. Péter Hegyi, M.D., Ph.D., D.Sc.[1,2]

Prof. Miklós Sahin-Tóth, M.D., Ph.D.[3]

[1]First Department of Medicine
Faculty of Medicine
University of Szeged

[2]MTA-SZTE Lendület Translational Gastroenterology Research Group

[2]Department of Molecular and Cell Biology
Boston University
Henry M. Goldman School of Dental Medicine

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INTRODUCTION

Genetic risk factors are important determinants of disease susceptibility and prognosis in **chronic pancreatitis (CP)** and **pancreatic ductal adenocarcinoma (PDAC)**. The pathogenesis of these disorders is complex; in the majority of patients the disease develops through the interaction of environmental and genetic risk factors. Hereditary forms represent a minority of patients, where solely a genetic etiology is apparent. To date, several highly penetrant susceptibility genes have been identified in hereditary pancreatitis and familial pancreatic cancer syndromes, while low penetrance genetic risk factors often accumulate and interact with lifestyle and environment in sporadic or idiopathic cases.

A genetic background for CP was first described in a pedigree with an autosomal dominant inheritance pattern in 1952. In 1996 Whitcomb et al. identified the protease serine 1 (*PRSS1*) gene encoding the human cationic trypsinogen as the first gene associated with CP. Using candidate gene approaches several pancreatitis associated variants have been discovered in genes encoding serine protease inhibitor Kazal type 1 (*SPINK1*), chymotrypsin C (*CTRC*), cystic fibrosis transmembrane conductance regulator (*CFTR*), carboxypeptidase A1 (CPA1) and carboxyl ester lipase (*CEL*), while genome-wide association studies identified numerous other susceptibility loci. Despite these recent advances, many patients with idiopathic CP do not carry mutations in any of the known susceptibility genes, suggesting the involvement of other yet unidentified genes.

CP is an established risk factor for pancreatic cancer. Indeed, hereditary CP patients harboring the p.R122H mutation in the *PRSS1* gene are at 50% lifetime risk for developing cancer. It has been reported that common variants are shared between CP and PDAC, suggesting the idea that these conditions not only have common environmental but also common genetic risk factors. About 5% of PDAC cases are familial, involving highly penetrant susceptibility genes, such *BRCA2*, *ATM*, and *PALB2*. The remaining 95% of cases considered to be ‘sporadic’ have a significant germline genetic component as well, and are estimated to represent up to 30% of pancreatic cancer susceptibility. Such common variants were first identified in the AB0 blood group gene. To date, five genome-wide association studies (GWAS) have described multiple susceptibility loci associated with the risk of pancreatic cancer. However, estimates of heritability suggest a large number of loci remain to be discovered.

In the field of pancreas genetics there is a clear need to further explore the complex genetic background. Functional analysis of the disease-associated variants provides a better

understanding of the molecular pathogenesis and may open up new treatment possibilities. The other challenge is to understand what variations mean for an individual patient and how to apply this knowledge for treatment decisions. Although in its infancy, there is a growing body of evidence suggesting that individualized therapies that are based upon the specific genetic alterations of an individual patient will soon be a reality. In this thesis genetic investigations regarding chronic pancreatitis and pancreatic cancer have been reviewed together with the description of the author's experimental data in order to gain a deeper comprehension of the pathophysiology of pancreatic diseases.

Genetic risk factors of chronic pancreatitis

CP is an irreversible, progressive inflammatory disease of the pancreas, characterized by morphological changes of the gland, exocrine and/or endocrine insufficiency and chronic abdominal pain. CP is most commonly associated with excessive alcohol consumption but other factors such as smoking, metabolic disturbances, anatomic abnormalities, autoimmunity and genetic variations have also been implicated. In a substantial proportion of patients the cause of the disease remains obscure; thus, up to 30% of all cases are classified as having idiopathic CP. Whether or not the disease becomes manifest depends on the individual combination of genetic predisposition and exogenous insults, particularly alcohol intake and smoking.

The fact that the majority of individuals with high alcohol intake does not develop alcoholic CP suggest a genetic basis for susceptibility as well, such as variations in *SPINK1*, *CTRC*, and *CFTR* genes. In idiopathic CP the genetic risk is more apparent. Comprehensive screenings of the major risk genes in larger cohorts revealed that 30-50% of idiopathic CP patients carry one or more known disease associated mutations. In hereditary pancreatitis heterozygous mutations of *PRSS1* are causative. There have been major discoveries about the mechanism of action of CP associated mutations, involving premature activation of digestive enzymes, endoplasmic reticulum stress and diminished ductal bicarbonate secretion.

Ductal secretion defect

Fluid and HCO_3^- secretion is a vital function of pancreatic ductal epithelium and is fundamental for the integrity of the tissue. One of the functional consequences of CP is the reduction in secretin-stimulated bicarbonate content in pancreatic juice. Impaired HCO_3^- secretion disrupts the physiological interaction between acinar and duct cells, resulting in decreased intraluminal pH, premature trypsinogen activation, impaired acinar secretion,

obstruction of the lumen with protein plugs and finally destruction of the parenchyma. Genetic defects of *CFTR* chloride channel can increase the risk for pancreatitis. Heterozygous CF-causing severe and mild *CFTR* variants increase the risk 2.9 and 4.5-fold respectively.

SLC26A6 anion transporter

Pancreatic bicarbonate secretion is not only dependent on *CFTR* but also on the solute-linked carrier 26 (SLC26) anion transporters, localized in the apical membrane of pancreatic ductal epithelium. Earlier studies revealed a direct molecular interaction between CFTR and two SLC26 exchangers, namely A3 and A6, which results in mutual upregulation of their transport activity. Notably, CF causing *CFTR* mutations that retain normal or substantial Cl^- conductance exhibited a severe defect in CFTR dependent Cl^-/HCO_3^- exchange activity. This indicates that impairment of the coupled bicarbonate transport mechanism is sufficient to damage pancreatic function even in the presence of CFTR Cl^- channel activity. On the basis of its localization and its function as a Cl^-/HCO_3^- exchanger, SLC26A6 has been proposed to be a major contributor to the apical HCO_3^- secretion in the pancreatic duct.

Genetic risk factors of pancreatic cancer

Carcinoma of the exocrine pancreas is a genetic disease that is caused by inherited and acquired mutations in specific cancer-associated genes. There is no effective screening available and advanced disease is commonly present at initial diagnosis. Established risk factors are cigarette smoking, chronic inflammation of the pancreas, diabetes mellitus and increased body mass. CP and PDAC share common environmental risk factors, such as smoking and heavy alcohol consumption and share some common genetic susceptibility factors as well. Advances in our understanding of the genes involved in the molecular pathogenesis of pancreatic cancer have provided insight into the progression of normal pancreatic ductal cells to noninvasive precursor lesions and to invasive carcinoma; and important implications for the development of chemoprevention and early detection strategies.

Cholecystokinin-B receptor

The gastrin/CCK-B receptor is a member of the G protein-coupled receptor superfamily, physiologically expressed in the human pancreas. Binding of gastrin or CCK triggers activation of multiple signal transduction pathways that relay mitogenic signals to the nucleus and promote cell proliferation. Numerous studies have shown that CCK-B receptor plays a significant role in carcinogenesis and tumor progression. An alternatively spliced mRNA

form of the receptor generated by retention of intron 4 (designated CCK-BRi4sv for intron 4-containing splice variant; also referred to as CCK-C receptor) was reported in various tumours, including pancreatic cancer. The resulting CCK-BRi4sv receptor protein exhibits constitutive activation of cell proliferation pathways. Smith et al. (2012) reported a common single nucleotide polymorphism (SNP) in the cholecystokinin-B receptor gene (*CCKBR*) as a risk factor for PDAC, which has not been observed in prior GWAS studies. The authors showed in a small cohort (51 cases and 39 controls) that variant c.811+32C>A (rs1800843) located in intron 4 of *CCKBR* increased PDAC risk and was also associated with poorer survival. Using immunohistochemistry, Smith et al. (2012) found that tumors with variant c.811+32C>A expressed CCK-BRi4sv receptor protein, suggesting that the variant might be directly responsible for intron retention.

AIMS

I. On the basis of its localization in the apical membrane of the pancreatic duct and its function as a Cl^-/HCO_3^- exchanger, SLC26A6 has been proposed to be a major contributor to the apical HCO_3^- secretion in the pancreatic duct. However, the role of genetic variations in *SLC26A6* has remained unexplored in CP. Therefore we aimed to investigate *SLC26A6* gene variants in CP.

Specific aims: in this study, we aimed to sequence the entire coding region of *SLC26A6* in 100 non-alcoholic CP cases. We aimed to further investigate the identified variants in Hungarian and German cohorts of non-alcoholic and alcoholic CP.

II. There are few known risk factors in pancreatic adenocarcinoma and a better understanding of the molecular pathogenesis is urgently needed. Therefore, we aimed to re-evaluate the role of *CCKBR* variant c.811+32C>A as a novel genetic prognostic marker.

Specific aims: in this study we had three objectives: (1) to replicate the association between variant c.811+32C>A and the risk for developing pancreatic cancer in an independent population, (2) to evaluate the impact of the variant on patient survival and (3) to examine the functional effect of the variant on pre-mRNA splicing.

PATIENTS AND METHODS OF GENETIC ANALYSIS OF SLC26A6 IN CHRONIC PANCREATITIS

Subjects and study design

All patients gave written informed consent for genetic analysis. The study included CP patients originating from Hungary (n = 106) and Germany (n = 361). According to etiology, patients were divided into alcoholic CP and non-alcoholic CP groups. Alcoholic CP was defined by consumption of more than 80g/d (man) ethanol or more than 60g/d (women) for at least two years. 99 Hungarian and 171 German control subjects were recruited from adult volunteers who considered themselves generally healthy.

DNA extraction and genotyping

Genomic DNA was isolated from whole blood. In a discovery cohort of 60 non-alcoholic Hungarian CP patients the entire coding sequence and adjacent intronic sequences were amplified and sequenced. Primers were designed according to the published sequence of the human *SLC26A6* gene (GenBank: NM_022911.2). PCR was performed in a total volume of 30 µl, which contained 0.5 U HotStarTaq DNA Polymerase, 1.5 mM Mg_2Cl, 0.2 mM dNTP, 0.5 µM primer and 10-50 ng genomic DNA.

Restriction fragment length polymorphism.

Genotyping of the p.V206M and c.1191C>A (p.P397=) variants in the Hungarian cohort was carried out by restriction fragment length polymorphism analysis. PCR was performed with primer sets 7 and 16, and the products were digested with *Nla*III and *Bmr*I restriction enzymes, respectively.

Statistical analysis

Quantitative variables were described as mean ± SD. We tested the significance of the differences between allele frequencies in cases and controls by Fisher's exact test and calculated *p*-values and odds ratios using GraphPad Prism v6.0a.

PATIENTS AND METHODS OF GENETIC ANALYSIS OF CCKBR VARIANT IN PANCREATIC CANCER

Study population

All participants gave written informed consent for genetic analysis. 122 cases with a confirmed diagnosis of PDAC were recruited from the Hungarian National Pancreas Registry. For each patient, information about gender, age at diagnosis, method of diagnosis and date of death or date of last follow-up was collected. 106 control subjects were recruited from adult volunteers who considered themselves generally.

DNA extraction and genotyping

Genomic DNA was isolated from 300 µl EDTA-blood. Primers were designed according to the genomic sequence of *CCKBR* on chromosome 11 (GenBank NC_000011.10). PCR was performed in a total volume of 30 µl, which contained 0.5 U HotStarTaq DNA Polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer and 10-50 ng genomic DNA.

Construction of expression plasmids harboring CCKBR minigenes

We designed *CCKBR* minigenes that contain intron 4 placed in the appropriate context of the full length coding DNA. *CCKBR* coding DNA (GenBank NM_176875.3) was custom synthesized and cloned into the pcDNA3.1(-) plasmid using XhoI and EcoRI restriction sites. To create *CCKBR* minigenes, a 584 nucleotide long fragment containing intron 4 with or without the c.811+32C>A variant was custom synthesized and cloned into the pcDNA3.1(-) *CCKBR* plasmid using BsrGI and BamHI restriction sites.

Construction of lentiviral vectors

The pWPI lentivirus vector plasmid and the packaging plasmids (psPAX2 and pMD2.G) were obtained from Didier Trono's laboratory through Addgene. First, CCKBR minigene templates were PCR amplified with Phusion Flash High-Fidelity PCR Master Mix. CCKBR minigene inserts were then subcloned into pWPI plasmid between PmeI and PacI restriction sites. The lentivirus production in HEK 293T packaging cells was performed as described previously. Viral preparations were titrated on HEK 293T cells.

Cell culture, transfection and viral transduction

Human embrionic kidney (HEK) 293T cells were transfected using 2 µg plasmid DNA and 10 µL Lipofectamine 2000 in 2 ml Opti-MEM Reduced Serum Medium. After 4 h of

incubation, cells were washed and the transfection medium was replaced with 2 ml DMEM. Cells were harvested 24 h after this medium change. Capan-1 pancreatic adenocarcinoma cells were transduced with viral supernatant at multiplicity of infections (MOIs) of four.

RNA extraction and reverse transcription

Total RNA was isolated from transfected cells using RNeasy Mini Kit. Two μg RNA was reverse transcribed in the presence of RNase inhibitor RNasin Plus.

Quantification of *CCKBR* expression and splicing

Real-time PCR reactions were performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) on an ABI PRISM 7000 Sequence Detection System platform. Relative expression was calculated using the comparative CT method (ΔΔCT method). Relative expression of splice variants was studied by using two different primer sets, one amplifying both the spliced and unspliced forms of *CCKBR* and the other amplifying the intron 4-retaining splice variant (*CCKB-Ri4sv*) only. For absolute quantification of total *CCKBR* and *CCK-BRi4sv* expression, we generated external calibration curves using serial dilutions of minigene plasmid templates. Using the calibration curves, copy numbers of total *CCKBR* and unspliced *CCK-BRi4sv* were determined and expressed as percent of total (spliced plus unspliced) *CCKBR* expression.

Statistical analysis

Quantitative variables were described as mean ± SE. To test the association between pancreatic cancer and genotype/allele frequencies we used two-tailed Fisher's exact test. Two-sided log rank test was used to compare the difference between survival of pancreatic cancer patients harboring the A-allele (A/A or A/C) with survival of those patients with the C/C genotype. All the analyses were performed with GraphPad Prism.

RESULTS OF THE SCL26A6 STUDY

In the Hungarian discovery cohort we included 55 adult and five pediatric patients with non-alcoholic CP. We sequenced 21 exons of *SLC26A6*. We found four common variants: a missense variant c.616G>A (p.V206M; *rs13324142*) in exon 6 and three intronic variants: c.23+71_103del in intron 1 (*rs72201074*); c.183-4C>A in intron 2 (*rs34368826*); and c.1134+32C>A in intron 9 (*rs3821876*) in complete linkage disequilibrium, indicating a

conserved haplotype. Subsequently, we determined the distribution of the p.V206M associated haplotype in the Hungarian cohort by genotyping the p.V206M variant in 46 subjects with alcoholic CP and 99 control subjects. When genotype-frequencies were compared, the distribution of the p.V206M variant did not show a statistically significant difference between patients and controls. We performed a replication study in a German cohort consisting of 202 subjects with non-alcoholic CP, 159 subjects with alcoholic CP and 171 controls. In 40 non-alcoholic CP cases the coding region was sequenced and the p.V206M associated haplotype was detected in five patients (three heterozygous and two homozygous). In agreement with our findings in the Hungarian cohort, the distribution of the p.V206M variant did not show a statistically significant difference between cases with alcoholic or non-alcoholic etiology and controls.

RESULTS OF THE CCKBR STUDY

Variant c.811+32C>A does not predict risk for PDAC

First, we attempted to replicate the published association between variant c.811+32C>A in intron 4 of *CCKBR* and the risk for developing pancreatic cancer. To this end, we sequenced this region of *CCKBR* in our Hungarian cohort and detected variant c.811+32C>A in 35 heterozygous and 5 homozygous cases (allele frequency 18.4%), and in 32 heterozygous and 3 homozygous controls (allele frequency 17.9%).

Variant c.811+32C>A does not predict survival in PDAC

To address the hypothesis that variant c.811+32C>A may have a prognostic relevance, we analyzed this variant in relation to patient survival. Median follow-up time was 334 days, 12.3% of the observations were censored. Median survival of cases with A/C and A/A genotypes was not significantly different from those with the CC genotype (257 days and 266 days, respectively; p=0.45), indicating that this variant does not modify survival of PDAC patients.

Variant c.811+32C>A does not affect splicing of intron 4 in *CCKBR*

To determine whether variant c.811+32C>A has an effect on pre-mRNA splicing; we have tested two different cell lines. HEK 293T cells were transfected with expression plasmids carrying *CCKBR* minigenes with or without the intron 4 variant and examined mRNA expression. We found that expression of the *CCK-BRi4sv* intron 4-retaining splice variant corresponded to about 10% of total *CCKBR* mRNA and was not different between cells transfected with minigenes with or without the c.811+32C>A variant. Since splicing factors

can be differently expressed in carcinoma cells, we have analyzed CCKBR splicing in Capan-1 pancreatic adenocarcinoma cells as well. In line with the data on HEK 293T cells, splicing was not affected by the variant c.811+32C>A.

DISCUSSION

Role of SLC26A6 variants in chronic pancreatitis

Pancreatic ductal HCO_3^- secretion is essential for the maintenance of tissue integrity, and is impaired in CP. In the present study we investigated the association of *SLC26A6* variants with CP, based on the crucial role of this candidate gene in the maintenance of ductal fluid and bicarbonate secretion. However, the *SLC26A6* variants we identified did not alter the risk for development of either alcoholic or non-alcoholic CP.

Studies on native pancreatic ducts isolated from Slc26a6$^{-/-}$ mice have been controversial regarding the effect on ductal fluid and bicarbonate secretion. Our group investigated two acute pancreatitis models in Slc26a6$^{-/-}$ mice, and we did not detect a difference in disease severity compared to the wild type animals (unpublished observations). On the other hand, Slc26a6$^{-/-}$ mice exhibit a high incidence of oxalate nephrolithiasis, due to defective intestinal oxalate secretion and urinary excretion. In an attempt to identify association with nephrolithiasis, *SLC26A6* variants were screened in familiar hyperoxaluria and primary hyperparathyroidism, but none of the variants increased disease risk. Notably, the authors also reported co-segregation of 3 intronic variations with p.V206M. Functional analysis of the p.V206M mutation in *Xenopus* oocyte expression studies revealed a 30% decrease of oxalate transport activity. However, the variant did not influence oxalate excretion in heterozygous carrier subjects. Surmising that heterozygosity would result in a 15% reduction in transport, this defect may not be sufficient to alter oxalate homeostasis.

In conclusion, in this study we tested the hypothesis that pancreatitis-associated mutations may be located in the *SLC26A6* gene encoding a pancreatic Cl^-/HCO_3^- transporter, which interacts with CFTR. We did not find association between genetic variants of *SLC26A6* and CP.

Role of *CCKBR* variant c.811+32C>A in pancreatic cancer

Identification of pancreatic cancer susceptibility genes is of outmost importance to define high-risk populations who may benefit from early detection by screening tests. Based on its role in pancreatic carcinogenesis and regulation of tumor growth *CCKBR* is a promising candidate for a susceptibility gene. Indeed, several somatic mutations were identified in

colorectal and gastric cancers that alter receptor activity, sensitization and localization. Some of these mutations are located in the third intracellular loop of the receptor, which plays a critical role in signal transduction. The same loop is altered by the tumor associated *CCK-BRi4sv* splice variant, which retains intron 4 and codes for an insertion of 69 additional amino acid residues that enhances receptor activity. The molecular basis for this alternative splicing has been explained by aberrant expression of certain auxiliary splicing factors in carcinoma cells that are necessary for the spliceosome assembly. Alternatively, Smith et al. (2012, 2014) proposed that the c.811+32C>A intronic variant in *CCKBR* can induce retention of intron 4 and thereby increase risk for the development of PDAC and also lead to poorer survival in carriers. In contrast, here we demonstrated that variant c.811+32C>A has no effect on *CCKBR* mRNA splicing, and it is not associated with increased risk for pancreatic cancer, nor with shorter survival in PDAC.

In conclusion, data presented here argue that intronic variant c.811+32C>A in *CCKBR* is not associated with PDAC risk or survival in a Hungarian cohort and does not alter splicing of the *CCKBR* pre-mRNA. Despite the fact that our study was not designed to detect a potentially small effect of variant c.811+32C>A on cancer risk and we did not take into account age and tumor stage at diagnosis when analyzing survival, our findings are convincingly self-consistent. Therefore, we propose that variant c.811+32C>A is functionally harmless and it should be considered a common polymorphism with no clinical significance. Finally, our results highlight the necessity for replication studies and the importance of functional testing of new genetic risk markers.

SUMMARY

Background: Genetic risk factors are important determinants of disease susceptibility and prognosis in chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC). In this thesis two genetic investigations were conducted to gain a deeper comprehension of the pathophysiology of these diseases.

I. Pancreatic ductal HCO_3^- secretion is critically dependent on the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) and the solute-linked carrier 26 member 6 anion transporter (SLC26A6). Deterioration of HCO_3^- secretion is observed in CP, and *CFTR* mutations increase CP risk. Therefore, *SLC26A6* is a reasonable candidate for a CP susceptibility gene, which has not been investigated in CP patients so far.

II. Single nucleotide polymorphism c.811+32C>A in intron 4 of the cholecystokinin-B receptor gene (*CCKBR*) was previously reported to correlate with higher PDAC risk and

poorer survival. The variant was suggested to induce retention of intron 4, resulting in a new spliceform with enhanced receptor activity. Our objective was to validate the c.811+32C>A variant as an emerging biomarker for pancreatic cancer risk and prognosis.

Patients and methods

I. As a first screening cohort, 106 subjects with CP and 99 control subjects with no pancreatic disease were recruited from the Hungarian National Pancreas Registry. In 60 non-alcoholic CP cases the entire *SLC26A6* coding region was sequenced. In the Hungarian cohort variants c.616G>A (p.V206M) and c.1191C>A (p.P397=) were further genotyped by restriction fragment length polymorphism analysis. In a German replication cohort all exons were sequenced in 40 non-alcoholic CP cases and variant c.616G>A (p.V206M) was further analyzed by sequencing in 321 CP cases and 171 controls.

II. We genotyped variant c.811+32C>A in 122 PDAC cases and 106 controls by sequencing and examined its association with cancer risk and patient survival. To test the functional effect of variant c.811+32C>A on pre-mRNA splicing, we transfected HEK 293T cells and Capan-1 cells with *CCKBR* minigenes.

Results

I. Sequencing of the entire coding region revealed four common variants: intronic variants c.23+78_110del, c.183-4C>A, c.1134+32C>A, and missense variant c.616G>A (p.V206M) which were found in linkage disequilibrium indicating a conserved haplotype. The distribution of the haplotype did not show a significant difference between patients and controls in the two cohorts. A synonymous variant c.1191C>A (p.P397=) and two intronic variants c.1248+9_20del and c.-10C>T were detected in single cases.

II. The allele frequency of the variant was similar between patients and controls (17.9% and 18.4%, respectively). Survival analysis showed no significant difference between median survival of patients with the C/C genotype (266 days) and patients with the A/C or A/A genotypes (257 days). *CCKBR* minigenes with or without variant c.811+32C>A exhibited no difference in expression of the intron-retaining splice variant.

Conclusion

I. Our data show that *SLC26A6* variants do not alter the risk for the development of CP.

II: These data indicate that variant c.811+32C>A in *CCKBR* does not have a significant impact on pancreatic cancer risk or survival.

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“You can’t wait for inspiration. You have to go after it with a club.” - Jack London