GENETIC INVESTIGATIONS IN CHRONIC PANCREATITIS AND PANCREATIC CANCER

Ph.D. Thesis

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Pancreas (2015 in press) **IF: 2.96**

II.) Anita Balázs, Claudia Ruffert, Eszter Hegyi, István Hritz, László Czakó, Tamás Takács, Zoltán Szepes, Balázs Csaba Németh, Judit Gervain, Ferenc Izbéki, Adrienn Halász, Dezső Kelemen, Richárd Szmola, János Novák, Stefan Crai, Anita Illés, Áron Vincze, Zsolt Molnár, Márta Varga, Barnabás Bod, Gyula Farkas Jr, János Sümegi, Attila Szepes, Zsolt Dubravcsik, Natália Lásztity, Andrea Párniczky, József Hamvas, Csilla Andorka, Gábor Veres, Zsolt Szentkereszty, Zoltán Rakonczay Jr, József Maléth, Miklós Sahin-Tóth, Jonas Rosendahl, Péter Hegyi "GENETIC ANALYSIS OF THE BICARBONATE SECRETING ANION EXCHANGER SLC26A6 IN CHRONIC PANCREATITIS"
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Publications not related to the subject of the thesis:

I.) Anita Balázs, Péter Hegyi "CYSTIC FIBROSIS-STYLE CHANGES IN THE EARLY PHASE OF PANCREATITIS"
Clinics and Research in Hepatology and Gastroenterology (2015)
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II.) József Maléth, **Anita Balázs,** Zsolt Balla, Balázs Kui, Máté Katona, Linda Judák, István Németh, Petra Pallagi, Lajos Vince Kemény, Zoltán Rakonczay Jr, Viktória Venglovecz, Imr Földesi, Zoltán Pető, Áron Somorácz, Katalin Borka, Ditta Perdomo, Gergely Lukacs, Mike A. Gray, Sylvio Monterisi, Manuela Zaccolo, Matthias Sendler, Julia Mayerle, Jens Kühn, Markus M. Lerch, Miklós Sahin-Tóth, Péter Hegyi. "ALCOHOL DISRUPTS LEVELS AND FUNCTION OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR TO PROMOTE DEVELOPMENT OF PANCREATITIS. "
Gastroenterology (2014) S0016-5085(14)01336-5. **IF: 16.72**

III.) József Maléth, Tamara Madácsy, Petra Pallagi, **Anita Balázs,** Viktoria Venglovecz, Zoltán Rakonczay, Péter Hegyi "PANCREATIC EPITHELIAL FLUID AND BICARBONATE SECRETION IS SIGNIFICANTLY ELEVATED IN THE ABSENCE OF PHERIPHERAL SEROTONIN"
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LIST OF ABBREVIATIONS

ATM	ataxia teleangiectasia mutated
BRCA2	breast cancer 2, early onset
CCKBR	cholecystokinin-B receptor
CCKBRi4sv	intron 4-containing splice variant of CCK-B receptor
CEL	carboxyl ester lipase
CDKN2A	cyclin-dependent kinase inhibitor 2A
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CPA1	carboxypeptidase A1
CP	chronic pancreatitis
CTRC	chymotrypsin C
PDAC	pancreatic ductal adenocarcinoma
PRSS1	protease serine 1, human cationic trypsinogen
SPINK1	serine protease inhibitor Kazal type 1
ER	endoplasmic reticulum
FAMMM	familial atypical multiple-mole melanoma
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GWAS	genome-wide association studies
KRAS	Kirsten rat sarcoma viral oncogene homolog
MMR	mismatch repair genes
MSH2	DNA mismatch repair protein
hMLH1	MutL homolog 1
PALB2	partner and localizer of *BRCA2*
PanIN	pancreatic intraepithelial neoplasia
SLC26	solute-linked carrier 26
SMAD4	mothers against DPP homolog 4
STAS	sulphate transporter and anti-sigma antagonist
STK11	serine/threonine kinase 11
TP53	tumor protein P53
OR	odds ratio
OS	overall survival

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[1]. In 1996 Whitcomb et al. identified the protease serine 1 (*PRSS1*)[2] gene encoding the human cationic trypsinogen as the first gene associated with CP[2]. Using candidate gene approaches several pancreatitis associated variants have been discovered in genes encoding serine protease inhibitor Kazal type 1 (*SPINK1*)[3], chymotrypsin C (*CTRC*)[4], cystic fibrosis transmembrane conductance regulator (*CFTR*)[5], carboxypeptidase A1 (CPA1)[6] and carboxyl ester lipase (*CEL*)[7], 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.

Chronic pancreatitis 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[8]. 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[9]. About 5% of PDAC cases are familial, involving highly penetrant susceptibility genes, such as breast cancer 2, early onset (*BRCA2*), ataxia teleangiectasia mutated (*ATM*), and partner and localizer of *BRCA2* (*PALB2*)[10]. 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[11]. Such common variants were first identified in the AB0 blood group gene[12]. To date, five genome-wide association studies (GWAS) have described multiple susceptibility loci associated with the risk of pancreatic cancer.[13–17] 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

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. The prevalence of CP is about 50/100.000. Patients experience substantial impairment in health-related quality of life[18], have an increased risk for developing pancreatic cancer, and face a markedly decreased life expectancy[19]. 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.

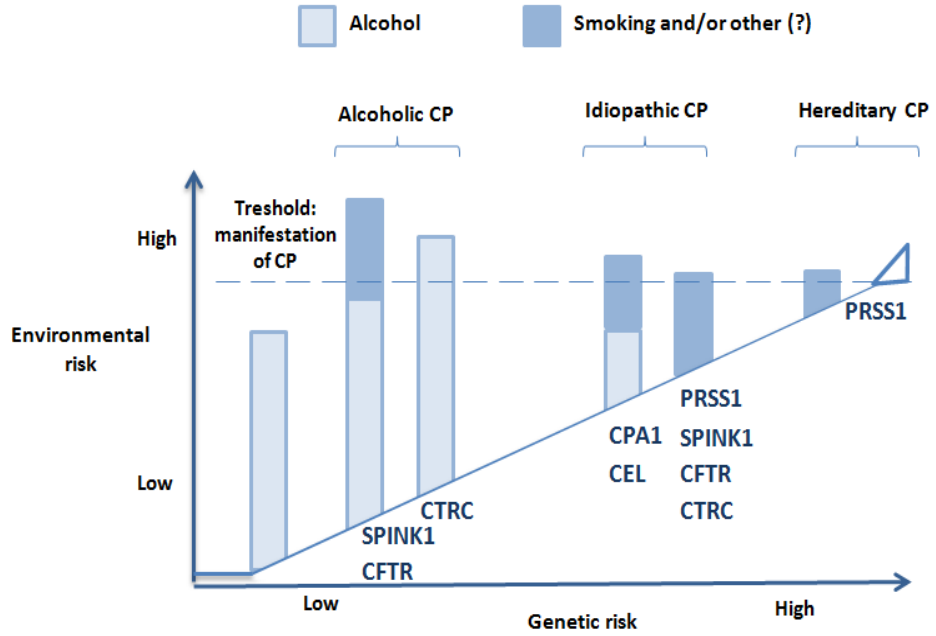

Figure 1.

Model of interaction between genetic susceptibility and environmental risk factors in chronic pancreatitis

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[20]. 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[21,22]. 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.

Protease-antiprotease imbalance

Tissue autodigestion in pancreatitis is a century-old paradigm. This pathogenic concept has been supported by several lines of genetic evidence, indicating that trypsin plays a key role in triggering an activation cascade of the digestive zymogens. Mutations in *PRSS1* cause increased autoactivation of mutant trypsinogens by directly increasing trypsinogen autoactivation or by altering the CTRC-dependent activation and/or degradation of cationic trypsinogen[23]. CTRC is a pancreatic serine protease which regulates autoactivation of cationic

trypsinogen by selectively cleaving regulatory sites within the activation peptide and the calcium binding loop. CP-associated mutations of *CTRC* cause impaired activity and/or decreased secretion, resulting in diminished trypsin-degrading activity[4]. Consistent with the trypsin-paradigm, a protective, loss-of-function variant of *PRSS2* encoding human anionic trypsinogen has been identified[24]. The *SPINK1* gene encodes the pancreatic secretory trypsin inhibitor, which is believed to play a part in protecting the pancreas against premature trypsinogen activation. The p.N34S mutation is the most frequent and best studied *SPINK1* variant, which is clearly a risk factor for CP, although the mechanism remains unclear[25]. It is plausible that intronic mutations in linkage disequilibrium with p.N34S may be responsible for the clinical effect. Taken together, gain-of-function mutations in PRSS1, or loss-of-function mutations in the trypsinogen-regulatory protease CTRC and protease inhibitor SPINK1 result in elevated intrapancreatic trypsin activity, leading to self-digestion of the gland.

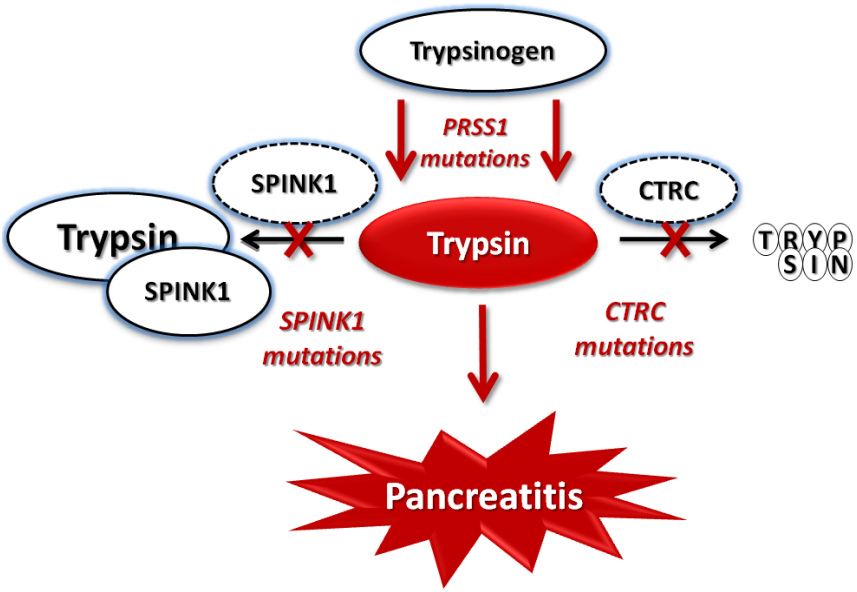

Figure 2.

Trypsin-central paradigm of pancreatitis.

Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a membrane-enclosed organelle responsible for the synthesis, folding, modification, and quality control of numerous secretory and membrane

proteins. The ER responds to the accumulation of unfolded proteins in its lumen (ER stress) by activating intracellular signal transduction pathways; cumulatively called the unfolded protein response (UPR). UPR increases the ER protein-folding capacity, reduces global protein synthesis, and enhances ER-associated degradation of misfolded proteins. Pancreatic acinar cells are particularly vulnerable to ER dysfunction, since they secrete enormous amount of proteins. First evidence that protein misfolding may be associated with CP came to light by investigation of several CP-associated *SPINK1* missense mutants, that exhibited a protein secretion defect [25]. Next, a hereditary CP associated *PRSS1* variant, namely p.R116C was found to induce misfolding and consequent ER stress, due to an unpaired cysteine residue[26]. A similar phenotype was observed for *PRSS1* variant p.C139S. Certain *CTRC* mutations also exhibited secretion defect and ER stress, that was proportional to the loss of secretion[27]. Mutations in *CPA1* were found to associate with CP, and the mechanism by which these variants confer increased pancreatitis risk was found involve misfolding-induced ER stress.[6] In a more recent study a carboxyl ester lipase hybrid allele (*CEL-HYB*) originating from a crossover between *CEL* and its neighboring pseudogene, *CELP* was found to increase susceptibility to idiopathic and alcoholic CP as well. Functional investigations revealed intracellular retention and impaired secretion.[7] Besides genetic evidence, ER stress was also observed in experimental models of pancreatitis, such as in the cerulein hyperstimulation model or the arginine-induced experimental acute pancreatitis model.[28]

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. The human pancreas secretes 1–2 liters of alkaline, isotonic juice per day, which contains concentrations of bicarbonate that may exceed 140mM[29]. This bicarbonate rich fluid flushes out digestive enzymes from the ductal tree, facilitates solubilization of macromolecules, neutralizes the protons secreted by acinar cells, prevents premature activation of trypsinogen and neutralizes gastric acid in the duodenum providing an optimal pH environment for digestive enzymes. One of the functional consequences of CP is the reduction in secretin-stimulated bicarbonate content in pancreatic juice[30]. 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.[31]. Several pancreatitis associated toxic factors, such as ethanol[32], bile acids[33], trypsin[34] and cigarette smoke extract[35] displayed inhibitory effects on bicarbonate secretion

and influenced the activity of the CFTR chloride channel. Not only functional inhibition, but also genetic defects of *CFTR* can increase the risk for pancreatitis. Association of *CFTR* mutations and the development of CP[5,36], and recurrent acute pancreatitis[38] has been reported. Genetic defects in the *CFTR* gene result in a wide disease spectrum (classic cystic fibrosis (CF), non-classic CF, CF-related diseases), which is dependent on organ-specific protein requirements, the amount of functional protein, - which is influenced by the genotype -, as well as genetic modifiers and environmental factors. *CFTR* variants found in CP patients are in most cases 'mild' variants with residual CFTR function, and it is yet unknown why heterozygote carriers are at increased disease risk. Heterozygous CF-causing severe and mild *CFTR* variants increase the risk 2.9 and 4.5-fold respectively[22]. These observations indicate that insufficient electrolyte transport is pathogenic for CP

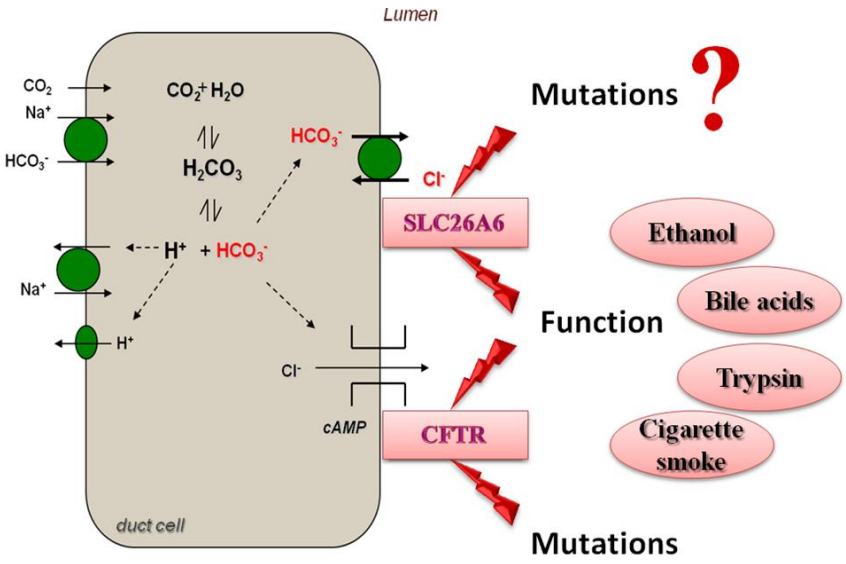

Figure 3.

Model of pancreatic ductal secretion.

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 the ductal cells. SLC26 isoforms constitute a conserved family of anion transporters with 10 distinct members. All SLC26 isoforms - except for SLC26A5 (prestin) - are versatile anion exchangers mediating apical chloride/base exchange in epithelial tissues. Several diseases have been linked to mutations in members of the family, including diastrophic dysplasias (SLC26A2), congenital chloride diarrhea (SLC26A3), Pendred's syndrome (SLC26A4), hearing loss

(SLC26A5) and asthma (SLC26A9)[39,40]. The SLC26A6 anion exchanger is expressed in the apical membrane of pancreatic ducts, intestinal epithelium and kidney proximal tubule[40]. It mediates multiple anion exchange modes, including, Cl^-/HCO_3^- exchange, Cl^-/formate exchange and Cl^-/oxalate exchange. 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[41]. This process is mediated by binding of the regulatory (R) domain of CFTR to the highly conserved STAS (sulphate transporter and anti-sigma antagonist) domain of SLC26 and this interaction is required for activation of both SLC26 transporters and CFTR. 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 [42]. 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[43]

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. It has the highest mortality rate of all malignancies. There is no effective screening available and advanced disease is commonly present at initial diagnosis.[44] The incidence of the disease is 8/100,000, and it is the fourth leading cause of cancer-related death among both men and women.[45] The majority of these tumors (85 percent) are adenocarcinomas arising from the ductal epithelium. Established risk factors are cigarette smoking, chronic inflammation of the pancreas, diabetes mellitus and increased body mass.[46] Chronic pancreatitis and PDAC share common environmental risk factors, such as smoking and heavy alcohol consumption and share some common genetic susceptibility factors as well. Cumulative risk for pancreatic cancer in CP reaches 1.8% at 10 years and 4% at 20 years, independent of the type of pancreatitis.[47] Hereditary pancreatitis is associated with a markedly increased, more than 50% lifetime risk for pancreatic cancer, although it accounts for a very small fraction of pancreatic cancer cases. The signaling mechanisms that underlie the transition from CP to invasive cancer involve inflammation induced transdifferentiation and oncogenic stimulation, resulting in metaplastic duct lesions or other precancerous lesions known as pancreatic intraepithelial neoplasia (PanIN). Eventually, PanIN can further progress to PDAC once cells acquire additional transforming mutations.[48]

Sequencing of pancreatic adenocarcinoma tissue samples revealed that multiple combinations of genetic mutations are commonly present that can be divided into three broad categories: mutational activation of oncogenes such as *KRAS;* inactivation of tumor suppressor genes such as *TP53*, p16/CDKN2A, and *SMAD4;* inactivation of genome maintenance genes, such as *hMLH1* and *MSH2*, which control the repair of DNA damage.[49] Although most of these genetic aberrations represent somatic mutations, others are present in the germline of kindreds who carry a familial predisposition to pancreatic cancer. 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.

High penetrance susceptibility genes

Pancreatic cancer aggregates in some families; an estimated 5-10% of individuals with pancreatic cancer have a family history of the disease. There are two broad categories of hereditary risk for pancreatic cancer: defined syndromes in which patients are at risk for a number of malignancies, including pancreatic cancer; and familial pancreatic cancer. Several high-penetrance germline mutations are involved in these cases, such as[50]:

- ***BRCA* and *PALB2*** - hereditary breast and ovarian cancer syndrome: Germline mutations in *BRCA1* and especially *BRCA2* are associated with an increased risk of pancreatic cancer. BRCA2 mutations are found in as many as 12-17% of patients with familial pancreatic cancer

- Germline mutations in ***STK11*** - Peutz-Jeghers syndrome. In individuals with PJS, the lifetime risk may be as high as 36%.

- Mutations in ***CDKN2A*** - Familial atypical multiple-mole melanoma (FAMMM) syndrome, a disorder associated with multiple nevi, cutaneous and ocular malignant melanomas, as well as pancreatic cancers.

- Ataxia-telangiectasia mutated (***ATM***) - Ataxia-telangiectasia is an autosomal recessive disorder associated with defective DNA repair mechanisms associated with an increased risk of pancreatic cancer

- Mismatch repair genes (**MMR**) - Lynch syndrome. Individuals with Lynch syndrome are also at increased risk of cancer of the ovary, stomach, pancreas, small bowel, hepatobiliary

system, transitional cell cancer of the renal pelvis and ureter, brain (glioma), and sebaceous neoplasms

- ***PRSS1*** mutations – hereditary pancreatitis and pancreatic cancer

However, combined, these known genetic factors account for less than 20% of the observed familial aggregation, suggesting that other as yet unidentified susceptibility genes may exist.

Low penetrance susceptibility genes

In sporadic cases of pancreatic cancer more common genetic variants are implicated that represent a minor risk for the disease. Epidemiologic evidence suggested that people with blood group 0 may have a lower risk of pancreatic cancer than those with groups A or B. Later risk variants in the AB0 blood group gene have been identified.[5,6] To date, five GWAS studies have described multiple susceptibility loci associated with the risk of pancreatic cancer.[13–17] Several of these loci harbor plausible candidate genes that have been implicated in pancreas development, pancreatic beta-cell function and predisposition to diabetes. The effect size of these susceptibility loci is generally small, with odds ratios (ORs) usually not higher than 1.2 or not lower than 0.8. When the cumulative association of risk alleles with pancreatic cancer was evaluated, it was found that compared to individuals with the most prevalent number (n = 10) of risk alleles in controls, those with ≤6 risk alleles had an OR of 0.55 (95% CI 0.44–0.68) and those with ≥14 risk alleles had an OR of 2.24 (95% CI 1.80–2.80) for pancreatic cancer[52].

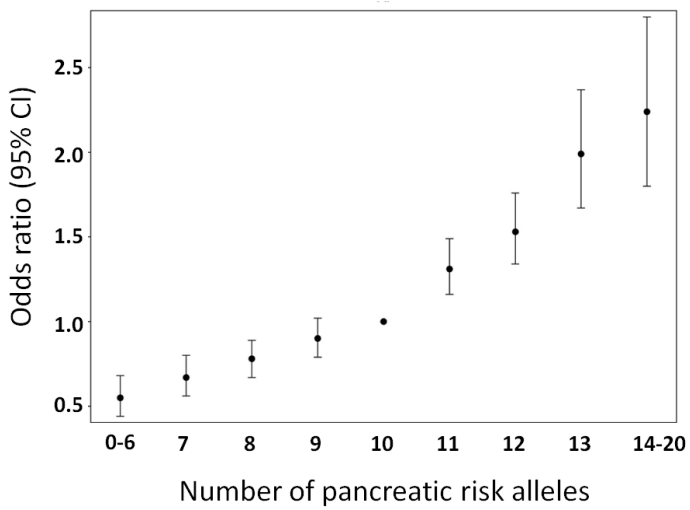

Figure 4. [52]

Cumulative association of risk alleles with pancreatic cancer

Cholecystokinin-B receptor

The gastrin/CCK-B receptor is a member of the G protein-coupled receptor superfamily, physiologically expressed in the human pancreas.[53] 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.[54,55] 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[56], including pancreatic cancer.[57] The resulting CCK-BRi4sv receptor protein exhibits constitutive (agonist-independent) activation of cell proliferation pathways.[58] 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.[59] 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. In a more recent follow-up study, Smith et al. (2014) replicated their results in a larger North-American multi-center cohort (931 cases and 59 controls) and confirmed both increased PDAC risk (odds ratio 2.28, CC versus AC plus AA genotypes) and shorter survival (hazard ratio 1.56) associated with variant c.811+32C>A.[60] 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. The authors speculated that binding of the splicing factor SRp55 might be reduced by the intronic variant resulting in enhanced retention of intron 4 [12, 13].

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 chronic pancreatitis.

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

The study protocol was approved by the national ethical review committee ETT-TUKEB (22254-1/2012). The study population has been collected and characterized by members of the Hungarian Pancreatic Study Group (see the list of coauthors). Therefore, characterization of study cohort which can be found in the manuscript is other coauthors scientific achievement. All patients gave written informed consent for genetic analysis. The study included CP patients originating from Hungary (n = 106) and Germany (n = 361). Clinico-pathological information on individual patients including symptoms, diagnostic criteria and etiology were collected from medical records and questioners completed by the patients. Diagnosis of CP was based on at least two of the following criteria: constant or recurrent abdominal pain, calcifications on sonography or CT, ductal irregularities on ERCP or MRCP examination, EUS based diagnosis of CP and histologically confirmed CP. 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, from inpatients who had no history of pancreatic disease and from blood donors.

DNA extraction and genotyping

Genomic DNA was isolated from whole blood using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). 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) (Table 1). Our analysis did not include the non-functional alternative splice variant SLC26A6d [61], which retains an unspliced intron, resulting in a different carboxy terminus lacking the STAS domain. PCR was performed in a total volume of 30 µl, which contained 0.5 U HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 1.5 mM Mg_2Cl, 0.2 mM dNTP, 0.5 µM primer and 10-50 ng genomic DNA. Amplification was performed under the following cycle conditions: 95 °C for 15 min to activate the enzyme, followed by 40

cycles of 30 s denaturation at 94 °C, 30 s at specific annealing temperatures and 1 min extension at 72 °C, with a final extension of 5 min. Prior to sequencing PCR products were visualized by agarose gel electrophoresis.

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, (see Table1) and the products were digested with *Nla*III (Thermo Scientific, Vilnius, Lithuania) and *Bmr*I (New England Biolabs, Ipswich, MA USA) 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 (San Diego, CA USA).

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

Study population

The study protocol has been approved by the Regional and Institutional Committee of Science and Research Ethics. The study population has been collected and characterized by members of the Hungarian Pancreatic Study Group (see the list of coauthors). Therefore, characterization of study cohort which can be found in the manuscript is other coauthors scientific achievement. 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. Two patients had synchronous or metachronous cancer suggestive of inherited cancer syndromes. Other cases were sporadic; no patients fulfilled the criteria for familial pancreatic cancer (two or more first degree relatives with pancreatic cancer). 106 control subjects were recruited from adult volunteers who considered themselves generally healthy and from inpatients, who had no history of pancreatic diseases. Tumor stage and survival of patients is described in Table 3.

DNA extraction and genotyping

Genomic DNA was isolated from 300 µl EDTA-blood using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). Primers were designed according to the genomic sequence of *CCKBR* on chromosome 11 (GenBank NC_000011.10) (see primer sequences in Table 4). PCR was performed in a total volume of 30 µl, which contained 0.5 U HotStarTaq DNA Polymerase (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer and 10-50 ng genomic DNA. Amplification was performed under the following cycle conditions: 95 °C for 15 min to activate the enzyme, followed by 40 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C and 1 min extension at 72 °C, with a final extension of 5 min. Prior to sequencing PCR products were purified with QIAquickPCR Purification Kit (Qiagen). Nucleotide sequence analysis was carried out in a commercial laboratory (Delta Bio 2000 Ltd., Szeged, Hungary) using a 3500 Genetic Analyser (Applied Biosystems) automatic dye-terminator sequencing machine. The reverse PCR primer was used as sequencing primer. Chromatograms were analyzed with ChromasPro software (Technelysium, South Brisbane, Australia).

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 (GenScript) 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.

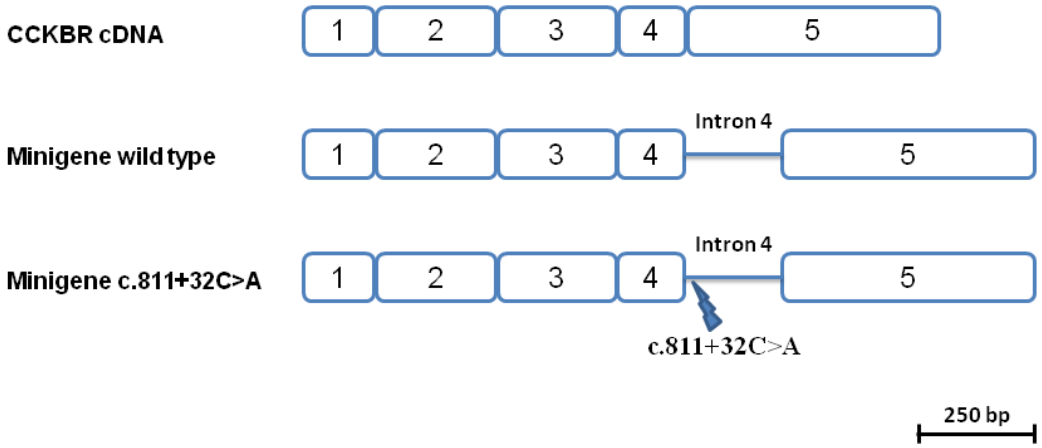

Figure 6. Minigene constructs used to analyze the effect of the c.811+32C>A variant on pre-mRNA splicing. Intron 4 was placed in the appropriate context of the *CCKBR* cDNA. Numbers indicate exons.

Construction of lentiviral vectors

The pWPI lentivirus vector plasmid and the packaging plasmids (psPAX2 and pMD2.G) were obtained from Didier Trono's laboratory (http://tronolab.epfl.ch/; Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) through Addgene (Addgene plasmids 12254, 12260, and 12259). First, CCKBR minigene templates were PCR amplified with Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) using the following primers 5'-GCTTAATTAACCATGGAGCTGCTAAAGCTGAACC-3' containing PacI restriction site and 5' phosphorilated 5'-CTCAGCCAGGGCCCAGTGTG-3'. 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[62]. Briefly, 293T cells were co-transfected with the pWPI expression plasmids, the packaging plasmid psPAX2 and the envelope vector pMD2.G. Transfection medium was changed after 16 h, and the lentivirus-containing medium was subsequently harvested after 48 hours and frozen at −80°C. Viral preparations were titrated on HEK 293T cells.

Cell culture, transfection and viral transduction

Human embrionic kidney (HEK) 293T cells were cultured in 6-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Budapest, Hungary) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37 °C in a humified atmosphere containing 5% CO_2. Transfections of HEK 293T cells were performed at 70-80%

confluence using 2 µg plasmid DNA and 10 µL Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) in 2 ml Opti-MEM Reduced Serum Medium (Life Technologies). 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 maintained in RPMI-1640 Medium (Sigma) supplemented with 15% fetal bovine serum 4 mM glutamine, and 1% penicillin/streptomycin at 37°C. To establish stable cell-lines a total number of 10^5 cells were plated in 6-well plates and transduced with viral supernatant at multiplicity of infections (MOIs) of four. Expression analysis was performed at first, second and third passages.

RNA extraction and reverse transcription

Total RNA was isolated from transfected cells using RNeasy Mini Kit (Qiagen). To avoid plasmid and genomic DNA contamination, an additional on-column DNase digestion step was applied with RNase-Free DNAse (Qiagen). Two µg RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in the presence of RNase inhibitor RNasin Plus (Promega, Fitchburg, WI, USA).

Quantification of *CCKBR* expression and splicing

Real-time PCR reactions were performed with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) platform with the following conditions: 10 min initial denaturation at 95 °C, followed by 40 two-step cycles: 15 s at 95 °C and 1 min at 60 °C. Primer sequences are given in Table 3. Threshold cycle (CT) values were determined using the 7000 Sequence Detection System Software V.1.2.3. Relative expression was calculated using the comparative CT method (ΔΔCT method). Expression level of *CCKBR* was first normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) internal control gene (ΔCT) and then to expression levels measured in cells transfected with empty vector (ΔΔCT). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$. 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. All reactions were performed in duplicates.

Statistical analysis

Quantitative variables were described as mean ± SE. Observed genotype frequencies in the study population were compared to the expected Hardy–Weinberg equilibrium. To test the association between pancreatic cancer and genotype/allele frequencies we used two-tailed Fisher's exact test. Additional odds ratios (OR) with 95% confidence interval (CI) were estimated. Overall survival (OS) was defined as the time interval between diagnosis and death (uncensored observation) or the last date when the patient was still known to be alive (censored observation). Survival curves were calculated for OS of patients according to Kaplan-Meier. 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. Median survival time was calculated using data from all patients; median follow-up time was computed with censored observations. All the analyses were performed with GraphPad Prism (San Diego, CA, USA). For sample size calculation we used Quanto v.1.2.4.[63].

RESULTS OF THE SCL26A6 STUDY

In the Hungarian discovery cohort we included 55 adult and five pediatric patients with non-alcoholic CP. No genetic testing was performed previously in the adult group. The pediatric patients were tested for *PRSS1*, *CTRC*, *SPINK1* and *CFTR* mutations and no pathogenic variants were found. We sequenced 21 exons of *SLC26A6*[64]. 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.

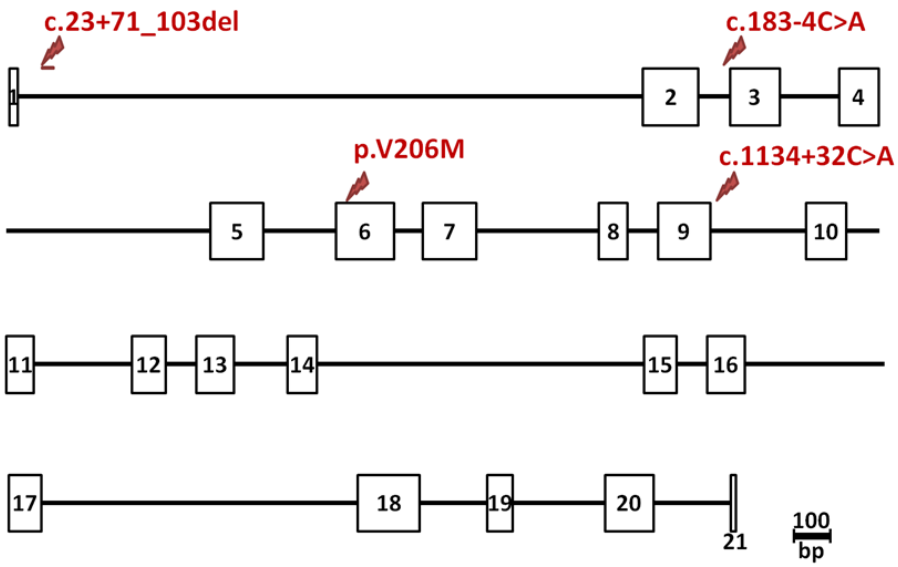

Figure 5. Schematic overview of the human *SLC26A6* gene. Squares represent exons. The p.V206M associated haplotype is indicated by red arrows.

One homozygous and heterozygous patients were identified with the haplotype (allele frequency 15.8%). One patient carried a synonymous mutation c.1191C>A (p.P397=) in exon 10 (*rs369278809*). 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. The Hungarian cohorts were also genotyped for the c.1191C>A (p.P397=) variant but beyond the single case identified by sequencing no additional carriers were found.

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). Additionally, two intronic variants c.1248+9_20del and c.-10C>T (*rs150438742*) were detected in single cases. 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 (Table 2a and b).

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%). Similarly to allele frequencies, genotype frequencies did not show a statistically significant difference between cases and controls either (Table 5). Genotype frequencies in cases and controls were found to conform to the Hardy-Weinberg equilibrium. Additionally, we identified two variants in exon 5: c.955C>T (p.R319W, rs113168010) in one control subject and c.956G>A (p.R319Q, rs1805001) in a single patient.

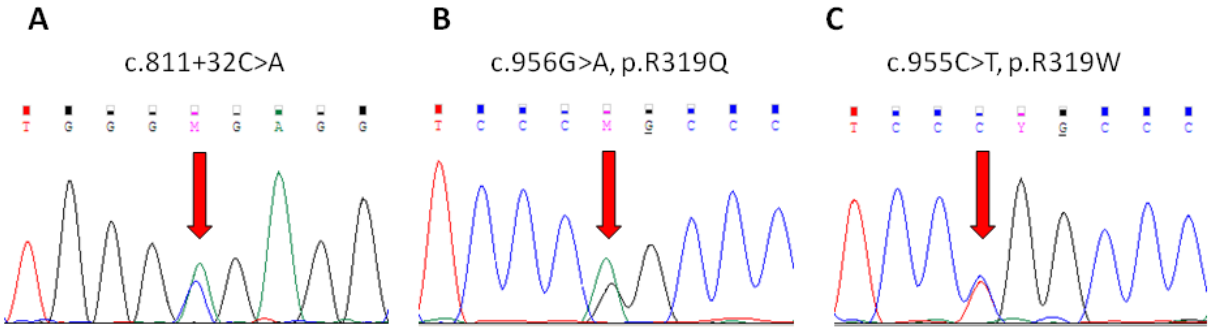

Figure 7. Sequence electropherograms of *CCKBR* gene variants found in our cohort.

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.

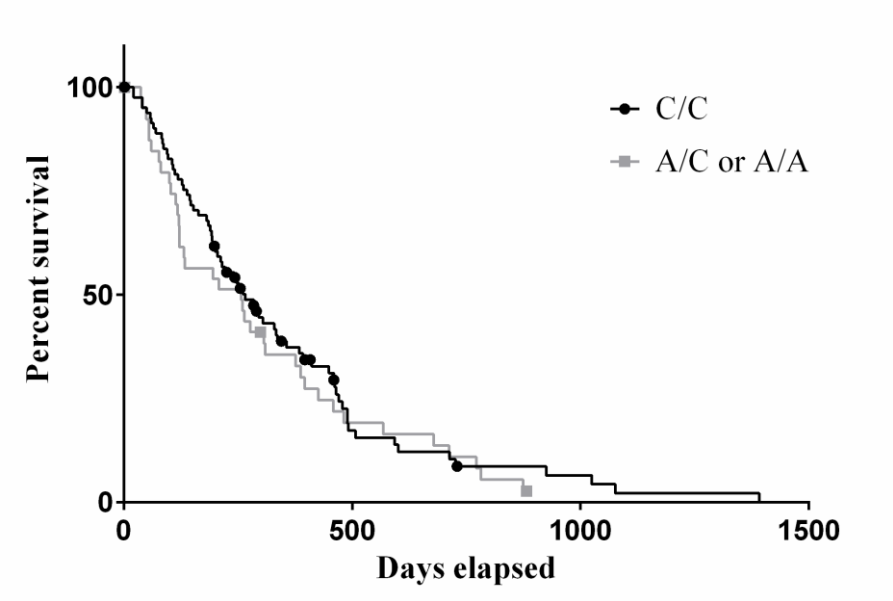

Figure 8.

Kaplan-Meier survival curves according to genotype.

Censored cases are shown as dots and squares.

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. *CCKBR* expression in transfected HEK 293T cells was about six orders of magnitude higher than endogenously expressed levels (Figure 8A). Interestingly, *CCKBR* mRNA was expressed at 1.5-fold higher levels when cells were transfected with intron-containing minigenes compared to cells transfected with the intronless *CCKBR* cDNA construct. This phenomenon is in agreement with published observations that the presence of introns can enhance gene expression.[65,66] For absolute quantification of different splice-forms, we generated calibration curves using minigene plasmids as template. 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 (Figure 8C).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 (Figure 8B, 8D).

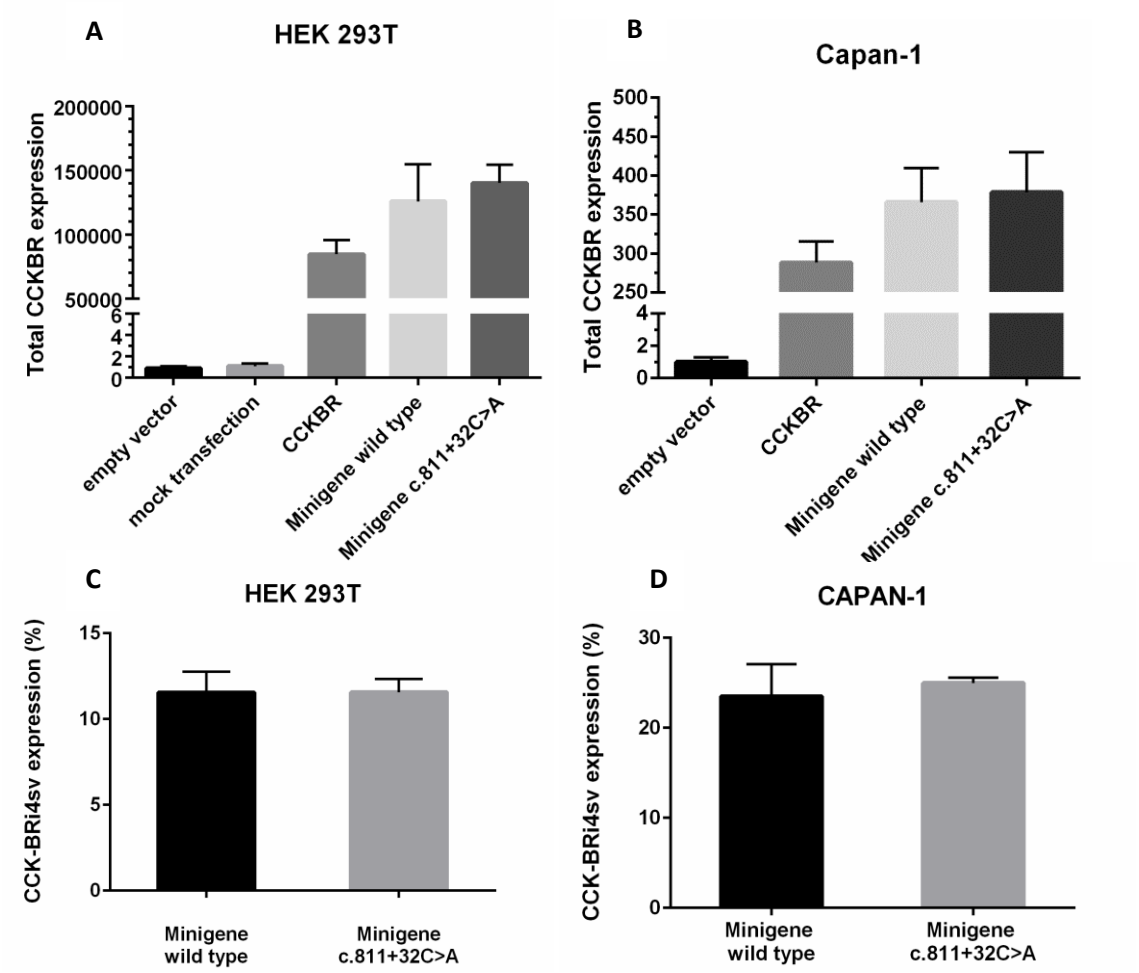

Figure 9. Functional analysis of the effect of variant c.811+32C>A on pre-mRNA splicing (**A**) Expression of *CCKBR* mRNA in transfected HEK 293T cells and transduced Capan-1 cells (**B**) with the indicated constructs. (**C**) Expression of the intron-retaining splice variant relative to the total amount of *CCKBR* mRNA. Results are from three independent transfections with seven parallels each.

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. Smoking represents an independent risk factor for the development of CP[67], possibly by impairing HCO_3^- transport[6869] Therefore, we compared the occurrence of the p.V206M variant in patients where data on smoking habits were available. It is conceivable, that mutations of the SLC26A6 anion transporter could influence the effects of smoking, however, we did not find a significant difference between genotype frequencies of smokers and non-smokers (data not shown). Previously, Amato et al. (2012) examined SLC26 anion transporter and epithelial Na^+ channel genes in 39 patients with CFTR related disorders, and found no association.[70] They described the *SLC26A6* variants which we also identified as the p.V206M associated haplotype, however, they did not report linkage of these variants.

Studies on native pancreatic ducts isolated from Slc26a6$^{-/-}$ mice have been controversial regarding the effect on ductal fluid and bicarbonate secretion[71,72]. 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[73], due to defective intestinal oxalate secretion and urinary excretion[74]. 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 [75,76] 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 [75]. Surmising that heterozygosity would result in a 15% reduction in transport, this defect may not be sufficient to alter oxalate homeostasis.

The STAS domain of SLC26A6 plays a key role in the functional interaction with CFTR[41]. The p.V206M mutation is located outside the STAS domain, and therefore, most likely does not have a substantial effect on bicarbonate transport.

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.[77,78] 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.[58] 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.[79] 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 [12, 13]. 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. Although we had more than 85% statistical power to replicate the previously described odds ratio of 2.28, we detected no enrichment of the variant in our PDAC cohort. The reasons for the discrepancy between our results and those of Smith et al. (2012, 2014) are not readily apparent but may be related to ethnic and geographic variability of the frequency of the c.811+32C>A variant and the admixed nature of the US cohort. Association studies in ethnically admixed populations are potentially vulnerable to spurious association due to the ethnic variability of the SNP frequency studied. Indeed, data retrieved from the 1000 Genome Project database (www.1000genomes.org) show that the allele frequency of variant c.811+32C>A is 18.4% in

subjects of European origin, whereas it is 2% in subjects of Asian descent and 23% in subjects of African descent. We also note that the control group in the study by Smith et al. (2014) was unusually small (59 subjects), which might result in the incorrect determination of control genotype frequencies. Indeed, the reported minor allele frequency (11.8%) for this control cohort is appreciably smaller than the incidence found in our controls (17.9%) which compares well with the 1000 Genomes data.

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 pancreatic adenocarcinoma 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.

TABLES

Table 1. Oligonucleotide primers and conditions used for PCR amplification and sequencing of the coding exons and the exon-intron junctions in the *SLC26A6* gene.

Exon	Primer name	Sequence (5′→3′)	PCR product (bp)	Annealing temperature (^{o}C)
Exon 1	Primer 01. F	TCCGGAGCGTAGCGGCCT	289	58.7 - 60
	Primer 01. R	GCACAGCCCAAGGGACTGG		
Exon 2-3	Primer 02. F	GATGCCTTCACTGTGTCTCTC	522	53.5 - 59.7
	Primer 02. R	CTGGGTTAGGTGCCATAGTTC		
Exon 4	Primer 03. F	ATCGTTTCAAGATCTGCTCTCC	214	53 – 59.7
	Primer 03. R	CCATGATGGATGTGGGCAT		
Exon 5	Primer 04. F	AGTGTCCTCTCCTCTTCAGAC	217	53 – 58.1
	Primer 04. R	CATACTCCTGACTGTTCCACAC		
Exon 6-7	Primer 05. F	CTGCGCTCCTCATTAGCAACC	538	65.1
	Primer 05. R	TACAGGAGGCTGCCCACGTGG		
Exon 8-9	Primer 06. F	CTCACCTCACAGTGGTTTATGT	704	52 – 60
	Primer 06. R	GATGCCTCCGATAAGGTTACTG		
Exon 10-11	Primer 07. F	TACAGTGGAACAGTGACCAGC	424	57 – 60.2
	Primer 07. R	CTCGCCTGAACCTAGACTGG		
Exon 12-13	Primer 08. F	GAGGAGGGTTGTCAGCATC	401	56.2 – 60.6
	Primer 08. R	CCCTGTGGTACTCTCTCACTA		
Exon 12-13	Primer 09. F	GGGACTTCAGGCTCCTTC	219	53 – 56.2
	Primer 09. R	CGAATCCACAAAGGCTCATTC		
Exon 14	Primer 10. F	GCAGGCACTGGGCACACTAGG	235	60 – 65.1
	Primer 10. R	GACCTGCTAGGGGAGTGAAGC		
Exon 15-16	Primer 11. F	ATTCCCTGTCTTCCCTGGTGTA	235	53 – 60.6
	Primer 11. R	CATCGGCGCAACACCCT		
Exon 17	Primer 12. F	TCCTGTCTTTGCACACCTATG	409	54.6 – 61
	Primer 12. R	GAGTGCTCTCAGGGCAAATTA		
Exon 18	Primer 13. F	CCCAAACCCTCAAAGCTC	207	56 – 61
	Primer 13. R	AAAGTATCCTACCCTCTTCCC		
Exon 19	Primer 14. F	GGAGTTGAGTTCCTAGAGGTTC	321	53 – 61
	Primer 14. R	CATGGCCACCAGGAAAGA		
Exon 20	Primer 15. F	TCTTTGGATAAAGCTGTTCTAGGG	200	57 – 58.6
	Primer 15. R	GGGACTCCTGGGTAGCA		
Exon 21	Primer 16 F	TCAATGAGACAGCCAGAGATGC	620	57-61
	Primer 16 R	CATTCAACAGCTTCACCACCAC		

Table 2. Distribution of the p.V206M *SLC26A6* variant (genotypes in Table 3A and allele frequencies in Table 3B) in non-alcoholic and alcoholic chronic pancreatitis patients and control subjects from Hungary and Germany. Calculations were performed for a recessive model (GG+GA vs. AA) and a dominant model (GG vs. GA+AA) using two-sided Fisher Exact test. *P*-values are displayed for the dominant model (no significant *p*-value was obtained for the recessive model).

Table 2A

Country	**Genotype**			***p*-Value**	**OR (95% CI)**
Hungary	**GG**	**GA**	**AA**		
Non-alcoholic CP (n=60)	43 (71.6%)	16 (26.6%)	1 (1.6%)	0.78	1.11 (0.54-2.28)
Alcoholic CP (n=46)	36 (78.2%)	9 (19.5%)	1 (2.2%)	0.56	0.78 (0.34-1.26)
Controls (n=99)	73 (73.7%)	24 (24.2%)	2 (2%)	n.a.	n.a.
Germany					
Non-alcoholic CP (n=202)	159 (78.7%)	36 (17.8%)	7 (3.4%)	0.57	0.87 (0.54-1.41)
Alcoholic CP (n=159)	128 (80.5%)	28 (17.6%)	3 (1.9%)	0.27	0.74 (0.44-1.26)
Controls (n=171)	129 (75.4%)	32 (18.7%)	10 (5.9%)	n.a.	n.a.

Table 2B

Country	**Allele**		***p*-Value**	**OR (95% CI)**
Hungary	***G***	***A***		
Non-alcoholic CP (n=120)	102 (85%)	18 (15%)	0.83	1.07 (0.56-2.03)
Alcoholic CP (n=92)	81 (88%)	11 (12%)	0.61	0.82 (0.39-1.74)
Controls (n=198)	170 (85.9%)	28 (14.1%)	n.a.	n.a.
Germany				
Non-alcoholic CP (n=404)	354 (87.6%)	50 (12.4%)	0.26	0.79 (0.52-1.20)
Alcoholic CP (n=318)	284 (89.3%)	34 (10.7%)	0.09	0.67 (0.42-1.06)
Controls (n=342)	290 (84.8%)	52 (15.2%)	n.a.	n.a.

Table 3. Tumor stage and survival of patients

	no. of cases	**median survival±SD, days**
localized	9	480±312
locally advanced	78	321±267
metastasized	29	222±204
unknown	5	

Table 4. Oligonucleotide primers used in this study.

Primers used for genotyping		
	forward	*5'-CTGTGTTGCCTTCAGGTCCG-3'*
	reverse	*5'-ATCACCAGCAACATTCGCAC-3'*
Primers used for RT-PCR		
CCKBR-total	forward	*5'-TCTCCTCAACAGCAGCAGTG-3'*
	reverse	*5'-CCCAGGACCACGATGATGAG-3'*
CCKB-Ri4sv	forward	*5'-AATGGAGTTGAGCTGGGAGC-3'*
	reverse	*5'-TGGGCGGTCAGAGAAAAAGG-3'*
GAPDH	forward	*5'-CACCATCTTCCAGGAGCGAG-3'*
	reverse	*5'- GACTCCACGACGTACTCAGC -3'*

Table 5. Genotype and allele frequencies of variant c.811+32C>A in PDAC patients and controls. OR, odds ratio; CI, confidence interval.

	PDAC cases	**Controls**	**genotypic OR (95% CI)**	**p value**
CC	82/122	71/106	Reference	-
AC	35/122	32/106	0.947 (0.5328-1.683)	0.884
AA	5/122	3/106	1.443 (0.339-6.255)	0.7271
AC+AA	40/122	35/106	0.9895 (0.5686-1.722)	1
			allelic OR (95% CI)	
Minor allele frequency	18.4%	17.9%	1.01 (0.58-1.76)	1

REFERENCES

1. Comfort, M. W. & Steinberg, A. G. Pedigree of a family with hereditary chronic relapsing pancreatitis. *Gastroenterology* **21,** 54–63 (1952).
2. Whitcomb, D. C. *et al.* Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat. Genet.* **14,** 141–145 (1996).
3. Witt, H. *et al.* Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat. Genet.* **25,** 213–216 (2000).
4. Rosendahl, J. *et al.* Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. *Nat. Genet.* **40,** 78–82 (2008).
5. Cohn, J. A. *et al.* Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. *N. Engl. J. Med.* **339,** 653–658 (1998).
6. Witt, H. *et al.* Variants in CPA1 are strongly associated with early onset chronic pancreatitis. *Nat. Genet.* **45,** 1216–1220 (2013).
7. Fjeld, K. *et al.* A recombined allele of the lipase gene CEL and its pseudogene CELP confers susceptibility to chronic pancreatitis. *Nat. Genet.* **47,** 518–522 (2015).
8. Howes, N. *et al.* Clinical and genetic characteristics of hereditary pancreatitis in Europe. *Clin. Gastroenterol. Hepatol.* **2,** 252–261 (2004).
9. Malats, N. *et al.* Genetic variants associated with PDAC also contribute to chronic pancreatitis susceptibility. *Pancreatology* **15,** S16 (2015).
10. Bartsch, D. K., Gress, T. M. & Langer, P. Familial pancreatic cancer--current knowledge. *Nat. Rev. Gastroenterol. Hepatol.* **9,** 445–453 (2012).
11. Lu, Y. *et al.* Most common 'sporadic' cancers have a significant germline genetic component. *Hum. Mol. Genet.* **23,** 6112–6118 (2014).
12. Marcus, D. M. The ABO and Lewis blood-group system. Immunochemistry, genetics and relation to human disease. *N. Engl. J. Med.* **280,** 994–1006 (1969).

13. Amundadottir, L. *et al.* Genome-wide association study identifies variants in the ABO locus associated with susceptibility to pancreatic cancer. *Nat. Genet.* **41,** 986–990 (2009).
14. Low, S.-K. *et al.* Genome-Wide Association Study of Pancreatic Cancer in Japanese Population. *PLoS ONE* **5,** e11824 (2010).
15. Wu, C. *et al.* Genome-wide association study identifies five loci associated with susceptibility to pancreatic cancer in Chinese populations. *Nat. Genet.* **44,** 62–66 (2012).
16. Petersen, G. M. *et al.* A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat. Genet.* **42,** 224–228 (2010).
17. Wolpin, B. M. *et al.* Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat. Genet.* **46,** 994–1000 (2014).
18. Wehler, M. *et al.* Factors associated with health-related quality of life in chronic pancreatitis. *Am. J. Gastroenterol.* **99,** 138–146 (2004).
19. Thuluvath, P. J., Imperio, D., Nair, S. & Cameron, J. L. Chronic pancreatitis. Long-term pain relief with or without surgery, cancer risk, and mortality. *J. Clin. Gastroenterol.* **36,** 159–165 (2003).
20. da Costa, M. Z. G. *et al.* Genetic Risk for Alcoholic Chronic Pancreatitis. *Int. J. Environ. Res. Public. Health* **8,** 2747–2757 (2011).
21. Keiles, S. & Kammesheidt, A. Identification of CFTR, PRSS1, and SPINK1 mutations in 381 patients with pancreatitis. *Pancreas* **33,** 221–227 (2006).
22. Rosendahl, J. *et al.* CFTR, SPINK1, CTRC and PRSS1 variants in chronic pancreatitis: is the role of mutated CFTR overestimated? *Gut* **62,** 582–592 (2013).
23. Németh, B. C. & Sahin-Tóth, M. Human cationic trypsinogen (PRSS1) variants and chronic pancreatitis. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **306,** G466–G473 (2014).
24. Witt, H. *et al.* A degradation-sensitive anionic trypsinogen (PRSS2) variant protects against chronic pancreatitis. *Nat. Genet.* **38,** 668–673 (2006).
25. Király, O., Wartmann, T. & Sahin-Tóth, M. Missense mutations in pancreatic secretory trypsin inhibitor (SPINK1) cause intracellular retention and degradation. *Gut* **56,** 1433–1438 (2007).

26. Kereszturi, É. *et al.* Hereditary pancreatitis caused by mutation induced misfolding of human cationic trypsinogen - a novel disease mechanism. *Hum. Mutat.* **30,** 575–582 (2009).
27. Beer, S. *et al.* Comprehensive functional analysis of chymotrypsin C (CTRC) variants reveals distinct loss-of-function mechanisms associated with pancreatitis risk. *Gut* **62,** 1616–1624 (2013).
28. Sah, R. P. *et al.* Endoplasmic reticulum stress is chronically activated in chronic pancreatitis. *J. Biol. Chem.* **289,** 27551–27561 (2014).
29. Domschke, S. *et al.* Inhibition by somatostatin of secretin-stimulated pancreatic secretion in man: a study with pure pancreatic juice. *Scand. J. Gastroenterol.* **12,** 59–63 (1977).
30. Denyer, M. E. & Cotton, P. B. Pure pancreatic juice studies in normal subjects and patients with chronic pancreatitis. *Gut* **20,** 89–97 (1979).
31. Hegyi, P. & Petersen, O. H. The exocrine pancreas: the acinar-ductal tango in physiology and pathophysiology. *Rev. Physiol. Biochem. Pharmacol.* **165,** 1–30 (2013).
32. Maléth, J. *et al.* Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. *Gastroenterology* **148,** 427–439.e16 (2015).
33. Venglovecz, V. *et al.* Effects of bile acids on pancreatic ductal bicarbonate secretion in guinea pig. *Gut* **57,** 1102–1112 (2008).
34. Pallagi, P. *et al.* Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl^- channels and luminal anion exchangers. *Gastroenterology* **141,** 2228–2239.e6 (2011).
35. Raju, S. V. *et al.* Cigarette smoke induces systemic defects in cystic fibrosis transmembrane conductance regulator function. *Am. J. Respir. Crit. Care Med.* **188,** 1321–1330 (2013).
36. LaRusch, J. *et al.* Mechanisms of CFTR Functional Variants That Impair Regulated Bicarbonate Permeation and Increase Risk for Pancreatitis but Not for Cystic Fibrosis. *PLoS Genet.* **10,** (2014).
37. Bishop, M. D. *et al.* The cystic fibrosis transmembrane conductance regulator gene and ion channel function in patients with idiopathic pancreatitis. *Hum. Genet.* **118,** 372–381 (2005).

38. Cavestro, G. M. *et al.* Connections between genetics and clinical data: Role of MCP-1, CFTR, and SPINK-1 in the setting of acute, acute recurrent, and chronic pancreatitis. *Am. J. Gastroenterol.* **105,** 199–206 (2010).

39. Anagnostopoulou, P. *et al.* SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation. *J. Clin. Invest.* **122,** 3629–3634 (2012).

40. Mount, D. B. & Romero, M. F. The SLC26 gene family of multifunctional anion exchangers. *Pflüg. Arch. Eur. J. Physiol.* **447,** 710–721 (2004).

41. Ko, S. B. H. *et al.* Gating of CFTR by the STAS domain of SLC26 transporters. *Nat. Cell Biol.* **6,** 343–350 (2004).

42. Choi, J. Y. *et al.* Aberrant CFTR-dependent HCO3- transport inmutations associated with cystic fibrosis. *Nature* **410,** 94–97 (2001).

43. Steward, M. C. & Ishiguro, H. Molecular and cellular regulation of pancreatic duct cell function. *Curr. Opin. Gastroenterol.* **25,** 447–453 (2009).

44. Hidalgo, M. Pancreatic Cancer. *N. Engl. J. Med.* **362,** 1605–1617 (2010).

45. Yadav, D., Timmons, L., Benson, J. T., Dierkhising, R. A. & Chari, S. T. Incidence, prevalence, and survival of chronic pancreatitis: a population-based study. *Am. J. Gastroenterol.* **106,** 2192–2199 (2011).

46. Lowenfels, A. B. & Maisonneuve, P. Epidemiology and risk factors for pancreatic cancer. *Best Pract. Res. Clin. Gastroenterol.* **20,** 197–209 (2006).

47. Ekbom, A. *et al.* Pancreatitis and pancreatic cancer: a population-based study. *J. Natl. Cancer Inst.* **86,** 625–627 (1994).

48. Liou, G.-Y. *et al.* Macrophage-secreted cytokines drive pancreatic acinar-to-ductal metaplasia through NF-κB and MMPs. *J. Cell Biol.* **202,** 563–577 (2013).

49. Abramson, M. A., Jazag, A., van der Zee, J. A. & Whang, E. E. The Molecular Biology of Pancreatic Cancer. *Gastrointest. Cancer Res. GCR* **1,** S7–S12 (2007).

50. Saiki, Y. & Horii, A. Molecular pathology of pancreatic cancer. *Pathol. Int.* **64,** 10–19 (2014).

51. Wolpin, B. M. *et al.* ABO Blood Group and the Risk of Pancreatic Cancer. *J. Natl. Cancer Inst.* **101,** 424–431 (2009).

52. Wolpin, B. M. *et al.* Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat. Genet.* **46,** 994–1000 (2014).

53. Rozengurt, E. & Walsh, J. H. Gastrin, CCK, signaling, and cancer. *Annu. Rev. Physiol.* **63,** 49–76 (2001).

54. Aly, A., Shulkes, A. & Baldwin, G. S. Gastrins, cholecystokinins and gastrointestinal cancer. *Biochim. Biophys. Acta* **1704,** 1–10 (2004).

55. Smith, J. P. & Solomon, T. E. Cholecystokinin and pancreatic cancer: the chicken or the egg? *Am. J. Physiol. Gastrointest. Liver Physiol.* **306,** G91–G101 (2014).

56. Körner, M., Waser, B., Reubi, J. C. & Miller, L. J. CCK(2) receptor splice variant with intron 4 retention in human gastrointestinal and lung tumours. *J. Cell. Mol. Med.* **14,** 933–943 (2010).

57. Smith, J. P. *et al.* Characterization of the CCK-C (cancer) receptor in human pancreatic cancer. *Int. J. Mol. Med.* **10,** 689–694 (2002).

58. Hellmich, M. R. *et al.* Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth. *J. Biol. Chem.* **275,** 32122–32128 (2000).

59. Smith, J. P. *et al.* A single nucleotide polymorphism of the cholecystokinin-B receptor predicts risk for pancreatic cancer. *Cancer Biol. Ther.* **13,** 164–174 (2012).

60. Smith, J. P. *et al.* Distribution of Cholecystokinin-B Receptor Genotype Between Patients With Pancreatic Cancer and Controls and Its Impact on Survival. *Pancreas* (2014). doi:10.1097/MPA.0000000000000263

61. Lohi, H. *et al.* Isoforms of SLC26A6 mediate anion transport and have functional PDZ interaction domains. *Am. J. Physiol. - Cell Physiol.* **284,** C769–C779 (2003).

62. Dawson, K. *et al.* MicroRNA29: a mechanistic contributor and potential biomarker in atrial fibrillation. *Circulation* **127,** 1466–1475, 1475e1–28 (2013).

63. García-Closas, M. & Lubin, J. H. Power and sample size calculations in case-control studies of gene-environment interactions: comments on different approaches. *Am. J. Epidemiol.* **149,** 689–692 (1999).

64. Waldegger, S. *et al.* Cloning and characterization of SLC26A6, a novel member of the solute carrier 26 gene family. *Genomics* **72,** 43–50 (2001).

65. Wiegand, H. L., Lu, S. & Cullen, B. R. Exon junction complexes mediate the enhancing effect of splicing on mRNA expression. *Proc. Natl. Acad. Sci.* **100,** 11327–11332 (2003).

66. Kereszturi, E., Kiraly, O. & Sahin-Toth, M. Minigene analysis of intronic variants in common SPINK1 haplotypes associated with chronic pancreatitis. *Gut* **58,** 545–549 (2009).

67. Maisonneuve, P. *et al.* Cigarette smoking accelerates progression of alcoholic chronic pancreatitis. *Gut* **54,** 510–514 (2005).

68. Kadiyala, V. *et al.* Cigarette smoking impairs pancreatic duct cell bicarbonate secretion. *JOP J. Pancreas* **14,** 31–38 (2013).

69. Kreindler, J. L., Jackson, A. D., Kemp, P. A., Bridges, R. J. & Danahay, H. Inhibition of chloride secretion in human bronchial epithelial cells by cigarette smoke extract. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288,** L894–902 (2005).

70. Amato, F. *et al.* Extensive Molecular Analysis of Patients Bearing CFTR-Related Disorders. *J. Mol. Diagn.* **14,** 81–89 (2012).

71. Wang, Y. *et al.* Slc26a6 regulates CFTR activity in vivo to determine pancreatic duct HCO3- secretion: relevance to cystic fibrosis. *EMBO J.* **25,** 5049–5057 (2006).

72. Song, Y., Ishiguro, H., Yamamoto, A., Jin, C. X. & Kondo, T. Effects of Slc26a6 deletion and CFTR inhibition on HCO3- secretion by mouse pancreatic duct. *J. Med. Investig. JMI* **56 Suppl,** 332–335 (2009).

73. Jiang, Z. *et al.* Calcium oxalate urolithiasis in mice lacking anion transporter Slc26a6. *Nat. Genet.* **38,** 474–478 (2006).

74. Freel, R. W., Hatch, M., Green, M. & Soleimani, M. Ileal oxalate absorption and urinary oxalate excretion are enhanced in Slc26a6 null mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* **290,** G719–728 (2006).

75. Monico, C. G. *et al.* Phenotypic and Functional Analysis of Human SLC26A6 Variants in Patients With Familial Hyperoxaluria and Calcium Oxalate Nephrolithiasis. *Am. J. Kidney Dis. Off. J. Natl. Kidney Found.* **52,** 1096–1103 (2008).

76. Corbetta, S. *et al.* Analysis of the 206M polymorphic variant of the SLC26A6 gene encoding a Cl-oxalate transporter in patients with primary hyperparathyroidism. *Eur. J. Endocrinol. Eur. Fed. Endocr. Soc.* **160,** 283–288 (2009).

77. Willard, M. D. *et al.* Somatic Mutations in CCK2R Alter Receptor Activity that Promote Oncogenic Phenotypes. *Mol. Cancer Res. MCR* **10,** 739–749 (2012).

78. Schmitz, F. *et al.* CCK-B/gastrin receptors in human colorectal cancer. *Eur. J. Clin. Invest.* **31,** 812–820 (2001).

79. Ding, W.-Q., Kuntz, S. M. & Miller, L. J. A misspliced form of the cholecystokinin-B/gastrin receptor in pancreatic carcinoma: role of reduced sellular U2AF35 and a suboptimal 3'-splicing site leading to retention of the fourth intron. *Cancer Res.* **62,** 947–952 (2002).

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

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Genetic analysis of the bicarbonate secreting anion exchanger SLC26A6 in chronic pancreatitis

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ABSTRACT

Background: 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 chronic pancreatitis (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.
Methods: 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.

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Results: 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.

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

Introduction

Chronic pancreatitis (CP) is an intractable inflammatory disease of the pancreas, leading to progressive and irreversible destruction of the parenchyma. In the majority of patients the etiology is complex, the disease results from the interaction of multiple risk factors. 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. Discoveries of association of CP with *PRSS1* [1], *SPINK1* [2] and *CTRC* [3] gene mutations suggest a central role for trypsin in the development of CP, while association with *CFTR* [4] variants highlight the importance of ductal changes contributing to disease development.

Fluid and HCO_3^- secretion is a vital function of pancreatic ductal epithelium and is fundamental for the integrity of the tissue. The human pancreas secretes 1–2 L of alkaline, isotonic juice per day, which contains concentrations of bicarbonate that may exceed 140 mM [5]. This bicarbonate rich fluid flushes out digestive enzymes from the ductal tree, facilitates solubilization of macromolecules, neutralizes the protons secreted by acinar cells, prevents premature activation of trypsinogen and neutralizes gastric acid in the duodenum providing an optimal pH environment for digestive enzymes.

One of the functional consequences of CP is the reduction in secretin-stimulated bicarbonate content in pancreatic juice [6]. On the other hand, in cystic fibrosis (CF), pancreatic HCO_3^- secretion is impaired. 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 [7]. Several pancreatitis associated toxic factors, such as ethanol [8], bile acids [9], trypsin [10] and cigarette smoke extract [11] displayed inhibitory effects on bicarbonate secretion and influenced the activity of the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR). Not only functional inhibition, but also genetic defects of *CFTR* can increase the risk for pancreatitis. Association of *CFTR* mutations and the development of CP [4,12,13], and recurrent acute pancreatitis [14] has been reported. CF-causing severe and mild *CFTR* variants increase the risk 2.9 and 4.5-fold respectively [15]. These observations indicate that insufficient electrolyte transport is pathogenic for CP (Fig. 1).

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 the ductal cells. SLC26 isoforms constitute a conserved family of anion transporters with 10 distinct members. All SLC26 isoforms - except for SLC26A5 (prestin) - are versatile anion exchangers mediating apical chloride/base exchange in epithelial tissues. Several diseases have been linked to mutations in members of the family, including diastrophic dysplasias (SLC26A2), congenital chloride diarrhea (SLC26A3), Pendred's syndrome (SLC26A4), hearing loss (SLC26A5) and asthma (SLC26A9) [16,17].

The SLC26A6 anion exchanger is expressed in the apical membrane of pancreatic ducts, intestinal epithelium and kidney proximal tubule [17]. It mediates multiple anion exchange modes, including, Cl^-/HCO_3^- exchange, Cl^-/formate exchange and Cl^-/oxalate exchange. 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 [18]. This process is mediated by binding of the regulatory (R) domain of CFTR to the highly conserved STAS (sulphate transporter and anti-sigma antagonist) domain of SLC26 and this interaction is required for activation of both SLC26 transporters and CFTR. 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 impairement of the coupled bicarbonate transport mechanism is sufficient to damage pancreatic function even in the presence of CFTR Cl^- channel activity [19]. 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 [20]. However, the role of genetic variations in *SLC26A6* has remained unexplored in CP.

In this study, we sequenced the entire coding region of *SLC26A6* in 100 non-alcoholic CP cases. The identified variants were further investigated in Hungarian and German cohorts of non-alcoholic and alcoholic CP.

Methods

Subjects and study design

The study protocol was approved by the national ethical review committee ETT-TUKEB (22254-1/2012). All patients gave written informed consent for genetic analysis. The study included CP patients originating from Hungary (n = 106) and Germany (n = 361). Clinico-pathological information on individual patients including symptoms, diagnostic criteria and etiology were collected from medical records and questioners completed by the patients. Diagnosis of CP was based on at least two of the following criteria: constant or recurrent abdominal pain, calcifications on sonography or CT, ductal irregularities on ERCP or MRCP examination, EUS based diagnosis of CP and histologically confirmed CP. According to etiology, patients were divided into alcoholic CP and non-alcoholic CP groups. Alcoholic CP was defined by consumption of more than 80 g/d (man) ethanol or more than 60 g/d (women) for at least two years. 99 Hungarian and 171 German control subjects were recruited from adult volunteers who considered themselves generally healthy, from inpatients who had no history of pancreatic disease and from blood donors (Table 1).

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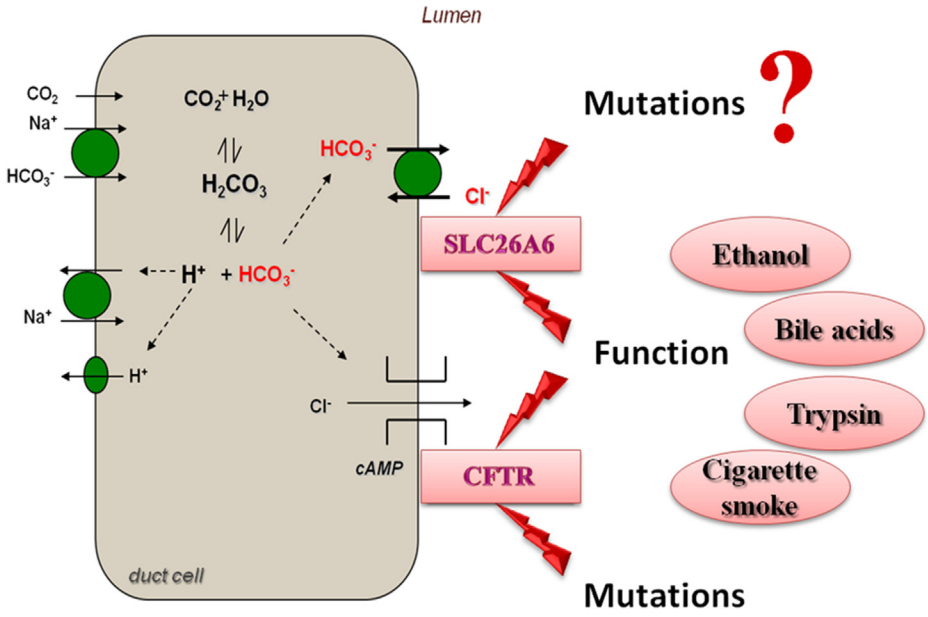

Fig. 1. Model of pancreatic ductal secretion. Bicarbonate secretion is dependent on the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) and the solute-linked carrier 26 member 6 (SLC26A6) anion transporter. Pancreatitis associated toxic factors such as ethanol, bile acids, trypsin and cigarette smoke alter bicarbonate secretion by inhibiting CFTR and SLC26A6. Genetic variants of CFTR are associated with CP. However, the role of genetic variations of the SLC26A6 anion transporter has not been investigated yet.

Polymerase chain reaction and DNA sequencing

Genomic DNA was isolated from whole blood using QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). In a discovery cohort of 60 non-alcoholic Hungarian CP patients and in 40 non-alcoholic German 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) (Table 2). Our analysis did not include the non-functional alternative splice variant SLC26A6d [21], which retains an unspliced intron, resulting in a different carboxy terminus lacking the STAS domain. PCR was performed in a total volume of 30 μl, which contained 0.5 U Hot-StarTaq DNA Polymerase (Qiagen, Hilden, Germany), 1.5 mM Mg_2Cl, 0.2 mM dNTP, 0.5 μM primer and 10–50 ng genomic DNA. Amplification was performed under the following cycle conditions: 95 °C for 15 min to activate the enzyme, followed by 40 cycles of 30 s denaturation at 94 °C, 30 s at specific annealing temperatures and 1 min extension at 72 °C, with a final extension of 5 min. Prior to sequencing PCR products were visualized by agarose gel electrophoresis. In the German cohort variant c.616G > A (p.V206M) was further analyzed by sequencing.

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, (see Table 1) and the products were digested with *Nla*III (Thermo Scientific, Vilnius, Lithuania) and *Bmr*I (New England Biolabs, Ipswich, MA USA) 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 (San Diego, CA USA).

Results

In the Hungarian discovery cohort we included 55 adult and five pediatric patients with non-alcoholic CP. No genetic testing was performed previously in the adult group. The pediatric patients were tested for *PRSS1*, *CTRC*, *SPINK1* and *CFTR* mutations and no

Table 1
Demographic data of cases and controls.

	Hungary		Germany	
	alcoholic CP (n = 46)	non-alcoholic CP (n = 60)	alcoholic CP (n = 159)	non-alcoholic CP (n = 202)
Age of onset range	19–74	3–85	18–73	3–75
Median age of onset ± SD	45.1 ± 10.9	44.7 ± 17.8	41.6 ± 11.2	33.2 ± 16.7
Male	41	35	137	100
Female	5	25	22	102
		Controls (n = 99)		Controls (n = 171)
Age		53.2 ± 17.7		63.8 ± 2.9
Male		43		83
female		53		88

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Table 2
Oligonucleotide primers and conditions used for PCR amplification and sequencing of the coding exons and the exon-intron junctions in the *SLC26A6* gene.

Exon	Primer name	Sequence (5′ → 3′)	PCR product (bp)	Annealing temperature (°C)
Exon 1	Primer 01. F	TCCGGAGCGTAGCGGCCT	289	58.7–60
	Primer 01. R	GCACAGCCCAAGGGACTGG		
Exon 2–3	Primer 02. F	GATGCCTTCACTGTGTCTCTC	522	53.5–59.7
	Primer 02. R	CTGGGTTAGGTGCCATAGTTC		
Exon 4	Primer 03. F	ATCGTTTCAAGATCTGCTCTCC	214	53–59.7
	Primer 03. R	CCATGATGGATGTGGGCAT		
Exon 5	Primer 04. F	AGTGTCCTCTCCTCTTCAGAC	217	53–58.1
	Primer 04. R	CATACTCCTGACTGTTCCACAC		
Exon 6–7	Primer 05. F	CTGCGCTCCTCATTAGCAACC	538	65.1
	Primer 05. R	TACAGGAGGCTGCCCACGTGG		
Exon 8–9	Primer 06. F	CTCACCTCACAGTGGTTTATGT	704	52–60
	Primer 06. R	GATGCCTCCGATAAGGTTACTG		
Exon 10–11	Primer 07. F	TACAGTGGAACAGTGACCAGC	424	57–60.2
	Primer 07. R	CTCGCCTGAACCTAGACTGG		
Exon 12–13	Primer 08. F	GAGGAGGGTTGTCAGCATC	401	56.2–60.6
	Primer 08. R	CCCTGTGGTACTCTCTCACTA		
Exon 12–13	Primer 09. F	GGGACTTCAGGCTCCTTC	219	53–56.2
	Primer 09. R	CGAATCCACAAAGGCTCATTC		
Exon 14	Primer 10. F	GCAGGCACTGGGCACACTAGG	235	60–65.1
	Primer 10. R	GACCTGCTAGGGGAGTGAAGC		
Exon 15–16	Primer 11. F	ATTCCCTGTCTTCCCTGGTGTA	235	53–60.6
	Primer 11. R	CATCGGCGCAACACCCT		
Exon 17	Primer 12. F	TCCTGTCTTTGCACACCTATG	409	54.6–61
	Primer 12. R	GAGTGCTCTCAGGGCAAATTA		
Exon 18	Primer 13. F	CCCAAACCCTCAAAGCTC	207	56–61
	Primer 13. R	AAAGTATCCTACCCTCTTCCC		
Exon 19	Primer 14. F	GGAGTTGAGTTCCTAGAGGTTC	321	53–61
	Primer 14. R	CATGGCCACCAGGAAAGA		
Exon 20	Primer 15. F	TCTTTGGATAAAGCTGTTCTAGGG	200	57–58.6
	Primer 15. R	GGGACTCCTGGGTAGCA		
Exon 21	Primer 16 F	TCAATGAGACAGCCAGAGATGC	620	57–61
	Primer 16 R	CATTCAACAGCTTCACCACCAC		

pathogenic variants were found. We sequenced 21 exons of *SLC26A6* (Fig. 2.) [22]. We found four common variants: a missense variant c.616G > A (p.V206M; *rs13324142*) in exon 6 and three intronic variants: c.23 + 78_110del in intron 1 (*rs556322139*); 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. One homozygous and heterozygous patients were identified with the haplotype (allele frequency 15.8%). One patient carried a synonymous mutation c.1191C > A (p.P397=) in exon 10 (*rs369278809*). 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 using RFLP. When genotype-frequencies were compared, the distribution of the p.V206M variant did not show a statistically significant difference between patients and controls. The Hungarian cohorts were also genotyped for the c.1191C > A (p.P397=) variant but beyond the single case identified by sequencing no additional carriers were found.

We performed a replication study in a German cohort consisting of 202 subjects with non-alcoholic CP, 159 subjects with alcoholic

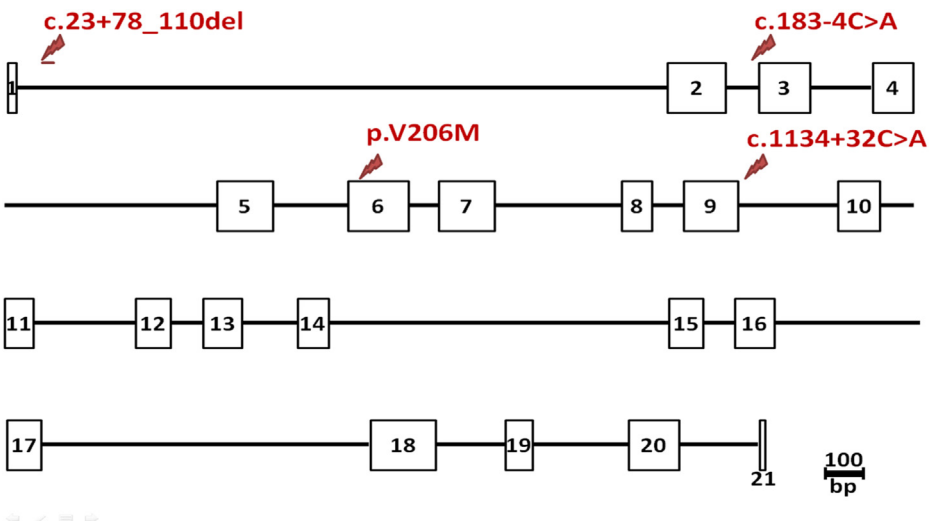

Fig. 2. Schematic overview of the human *SLC26A6* gene. Squares represent exons. The p.V206M associated haplotype is indicated by red arrows.

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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). Additionally, two intronic variants c.1248 + 9_20del and c.-10C > T (*rs150438742*) were detected in single cases. The rest of the German cohort was genotyped for variant p.V206M by sequencing. 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 (Table 3a and b). In order to exclude the possibility of interactions between pancreatitis susceptibility genes, one of the cohorts (German patients) were screened for risk variants in *PRSS1*, *CFTR*, *SPINK1*, and *CPA1* genes. Importantly, no variants were detected in linkage disequilibrium with p.V206M (data not shown).

Discussion

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. Smoking represents an independent risk factor for the development of CP [23], possibly by impairing HCO_3^- transport [24,25] Therefore, we compared the occurrence of the p.V206M variant in patients where data on smoking habits were available. It is conceivable, that mutations of the SLC26A6 anion transporter could influence the effects of smoking, however, we did not find a significant difference between genotype frequencies of smokers and non-smokers (data not shown). Previously, Amato et al. (2012) examined SLC26 anion transporter and epithelial Na^+ channel genes in 39 patients with CFTR related disorders, and found no association [26]. They described the *SLC26A6* variants which we also identified as the p.V206M associated haplotype, however, they did not report linkage of these variants.

Studies on native pancreatic ducts isolated from Slc26a6$^{-/-}$ mice have been controversial regarding the effect on ductal fluid and bicarbonate secretion [27,28]. 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 [29], due to defective intestinal oxalate secretion and urinary excretion [30]. 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 [31,32] 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 [31]. Surmising that heterozygosity would result in a 15% reduction in transport, this defect may not be sufficient to alter oxalate homeostasis.

The STAS domain of SLC26A6 plays a key role in the functional interaction with CFTR [18]. The p.V206M mutation is located outside the STAS domain, and therefore, most likely does not have a substantial effect on bicarbonate transport.

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 in Hungarian and German cohorts.

Table 3
Distribution of the p.V206M *SLC26A6* variant (genotypes in Table 3A and allele frequencies in Table 3B) in non-alcoholic and alcoholic chronic pancreatitis patients and control subjects from Hungary and Germany. Calculations were performed for a recessive model (GG + GA vs. AA) and a dominant model (GG vs. GA + AA) using two-sided Fisher Exact test. *P*-values are displayed for the dominant model (no significant *p*-value was obtained for the recessive model).

A

Country	Genotype			*p*-Value	OR (95% CI)
	GG	GA	AA		
Hungary					
Non-alcoholic CP (n = 60)	43 (71.6%)	16 (26.6%)	1 (1.6%)	0.78	1.11 (0.54–2.28)
Alcoholic CP (n = 46)	36 (78.2%)	9 (19.5%)	1 (2.2%)	0.56	0.78 (0.34–1.26)
Controls (n = 99)	73 (73.7%)	24 (24.2%)	2 (2%)	n.a.	n.a.
Germany					
Non-alcoholic CP (n = 202)	159 (78.7%)	36 (17.8%)	7 (3.4%)	0.57	0.87 (0.54–1.41)
Alcoholic CP (n = 159)	128 (80.5%)	28 (17.6%)	3 (1.9%)	0.27	0.74 (0.44–1.26)
Controls (n = 171)	129 (75.4%)	32 (18.7%)	10 (5.9%)	n.a.	n.a.

B

Country	Allele		*p*-Value	OR (95% CI)
	G	*A*		
Hungary				
Non-alcoholic CP (n = 120)	102 (85%)	18 (15%)	0.83	1.07 (0.56–2.03)
Alcoholic CP (n = 92)	81 (88%)	11 (12%)	0.61	0.82 (0.39–1.74)
Controls (n = 198)	170 (85.9%)	28 (14.1%)	n.a.	n.a.
Germany				
Non-alcoholic CP (n = 404)	354 (87.6%)	50 (12.4%)	0.26	0.79 (0.52–1.20)
Alcoholic CP (n = 318)	284 (89.3%)	34 (10.7%)	0.09	0.67 (0.42–1.06)
Controls (n = 342)	290 (84.8%)	52 (15.2%)	n.a.	n.a.

Recessive model.
p-value = 0.87; OR = 0.82; 95%CI = 0.73–9.27.
p-value = 0.95; OR = 1.08; 95%CI = 0.09–12.2.
p-value = 0.28; OR = 0.58; 95%CI = 0.22–1.56.
p-value = 0.08; OR = 0.31; 95%CI = 0.08–1.15.

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References

[1] Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, et al. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet 1996;14:141–5. http://dx.doi.org/10.1038/ng1096-141.

[2] Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. Nat Genet 2000;25:213–6. http://dx.doi.org/10.1038/76088.

[3] Rosendahl J, Witt H, Szmola R, Bhatia E, Ozsvári B, Landt O, et al. Chymotrypsin C (CTRC) variants that diminish activity or secretion are associated with chronic pancreatitis. Nat Genet 2008;40:78–82. http://dx.doi.org/10.1038/ng.2007.44.

[4] Cohn JA, Friedman KJ, Noone PG, Knowles MR, Silverman LM, Jowell PS. Relation between mutations of the cystic fibrosis gene and idiopathic pancreatitis. N Engl J Med 1998;339:653–8. http://dx.doi.org/10.1056/NEJM199809033391002.

[5] Domschke S, Domschke W, Rösch W, Konturek SJ, Sprügel W, Mitznegg P, et al. Inhibition by somatostatin of secretin-stimulated pancreatic secretion in man: a study with pure pancreatic juice. Scand J Gastroenterol 1977;12:59–63.

[6] Denyer ME, Cotton PB. Pure pancreatic juice studies in normal subjects and patients with chronic pancreatitis. Gut 1979;20:89–97.

[7] Hegyi P, Petersen OH. The exocrine pancreas: the acinar-ductal tango in physiology and pathophysiology. Rev Physiol Biochem Pharmacol 2013;165:1–30. http://dx.doi.org/10.1007/112_2013_14.

[8] Maléth J, Balázs A, Pallagi P, Balla Z, Kui B, Katona M, et al. Alcohol disrupts levels and function of the cystic fibrosis transmembrane conductance regulator to promote development of pancreatitis. Gastroenterology 2015;148:427–39. http://dx.doi.org/10.1053/j.gastro.2014.11.002. e16.

[9] Venglovecz V, Rakonczay Jr Z, Ozsvári B, Takács T, Lonovics J, Varró A, et al. Effects of bile acids on pancreatic ductal bicarbonate secretion in guinea pig. Gut 2008;57:1102–12. http://dx.doi.org/10.1136/gut.2007.134361.

[10] Pallagi P, Venglovecz V, Rakonczay Jr Z, Borka K, Korompay A, Ozsvári B, et al. Trypsin reduces pancreatic ductal bicarbonate secretion by inhibiting CFTR Cl^- channels and luminal anion exchangers. Gastroenterology 2011;141:2228–39. http://dx.doi.org/10.1053/j.gastro.2011.08.039. e6.

[11] Raju SV, Jackson PL, Courville CA, McNicholas CM, Sloane PA, Sabbatini G, et al. Cigarette smoke induces systemic defects in cystic fibrosis transmembrane conductance regulator function. Am J Respir Crit Care Med 2013;188:1321–30. http://dx.doi.org/10.1164/rccm.201304-0733OC.

[12] LaRusch J, Jung J, General IJ, Lewis MD, Park HW, Brand RE, et al. Mechanisms of CFTR functional variants that impair regulated bicarbonate permeation and increase risk for pancreatitis but not for cystic fibrosis. PLoS Genet 2014;10. http://dx.doi.org/10.1371/journal.pgen.1004376.

[13] Bishop MD, Freedman SD, Zielenski J, Ahmed N, Dupuis A, Martin S, et al. The cystic fibrosis transmembrane conductance regulator gene and ion channel function in patients with idiopathic pancreatitis. Hum Genet 2005;118:372–81. http://dx.doi.org/10.1007/s00439-005-0059-z.

[14] Cavestro GM, Zuppardo RA, Bertolini S, Sereni G, Frulloni L, Okolicsanyi S, et al. Connections between genetics and clinical data: role of MCP-1, CFTR, and SPINK-1 in the setting of acute, acute recurrent, and chronic pancreatitis. Am J Gastroenterol 2010;105:199–206. http://dx.doi.org/10.1038/ajg.2009.611.

[15] Rosendahl J, Landt O, Bernadova J, Kovacs P, Teich N, Bödeker H, et al. CFTR, SPINK1, CTRC and PRSS1 variants in chronic pancreatitis: is the role of mutated CFTR overestimated? Gut 2013;62:582–92. http://dx.doi.org/10.1136/gutjnl-2011-300645.

[16] Anagnostopoulou P, Riederer B, Duerr J, Michel S, Binia A, Agrawal R, et al. SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation. J Clin Invest 2012;122:3629–34. http://dx.doi.org/10.1172/JCI60429.

[17] Mount DB, Romero MF. The SLC26 gene family of multifunctional anion exchangers. Pflüg Arch Eur J Physiol 2004;447:710–21. http://dx.doi.org/10.1007/s00424-003-1090-3.

[18] Ko SBH, Zeng W, Dorwart MR, Luo X, Kim KH, Millen L, et al. Gating of CFTR by the STAS domain of SLC26 transporters. Nat Cell Biol 2004;6:343–50. http://dx.doi.org/10.1038/ncb1115.

[19] Choi JY, Muallem D, Kiselyov K, Lee MG, Thomas PJ, Muallem S. Aberrant CFTR-dependent HCO3- transport inmutations associated with cystic fibrosis. Nature 2001;410:94–7. http://dx.doi.org/10.1038/35065099.

[20] Steward MC, Ishiguro H. Molecular and cellular regulation of pancreatic duct cell function. Curr Opin Gastroenterol 2009;25:447–53. http://dx.doi.org/10.1097/MOG.0b013e32832e06ce.

[21] Lohi H, Lamprecht G, Markovich D, Heil A, Kujala M, Seidler U, et al. Isoforms of SLC26A6 mediate anion transport and have functional PDZ interaction domains. Am J Physiol - Cell Physiol 2003;284:C769–79. http://dx.doi.org/10.1152/ajpcell.00270.2002.

[22] Waldegger S, Moschen I, Ramirez A, Smith RJ, Ayadi H, Lang F, et al. Cloning and characterization of SLC26A6, a novel member of the solute carrier 26 gene family. Genomics 2001;72:43–50. http://dx.doi.org/10.1006/geno.2000.6445.

[23] Maisonneuve P, Lowenfels AB, Müllhaupt B, Cavallini G, Lankisch PG, Andersen JR, et al. Cigarette smoking accelerates progression of alcoholic chronic pancreatitis. Gut 2005;54:510–4. http://dx.doi.org/10.1136/gut.2004.039263.

[24] Kadiyala V, Lee LS, Banks PA, Suleiman S, Paulo JA, Wang W, et al. Cigarette smoking impairs pancreatic duct cell bicarbonate secretion. JOP J Pancreas 2013;14:31–8.

[25] Kreindler JL, Jackson AD, Kemp PA, Bridges RJ, Danahay H. Inhibition of chloride secretion in human bronchial epithelial cells by cigarette smoke extract. Am J Physiol Lung Cell Mol Physiol 2005;288:L894–902. http://dx.doi.org/10.1152/ajplung.00376.2004.

[26] Amato F, Bellia C, Cardillo G, Castaldo G, Ciaccio M, Elce A, et al. Extensive molecular analysis of patients bearing CFTR-related disorders. J Mol Diagn 2012;14:81–9. http://dx.doi.org/10.1016/j.jmoldx.2011.09.001.

[27] Wang Y, Soyombo AA, Shcheynikov N, Zeng W, Dorwart M, Marino CR, et al. Slc26a6 regulates CFTR activity in vivo to determine pancreatic duct HCO3- secretion: relevance to cystic fibrosis. EMBO J 2006;25:5049–57. http://dx.doi.org/10.1038/sj.emboj.7601387.

[28] Song Y, Ishiguro H, Yamamoto A, Jin CX, Kondo T. Effects of Slc26a6 deletion and CFTR inhibition on HCO3- secretion by mouse pancreatic duct. J Med Investig JMI 2009;56(Suppl. 332–5).

[29] Jiang Z, Asplin JR, Evan AP, Rajendran VM, Velazquez H, Nottoli TP, et al. Calcium oxalate urolithiasis in mice lacking anion transporter Slc26a6. Nat Genet 2006;38:474–8. http://dx.doi.org/10.1038/ng1762.

[30] Freel RW, Hatch M, Green M, Soleimani M. Ileal oxalate absorption and urinary oxalate excretion are enhanced in Slc26a6 null mice. Am J Physiol Gastrointest Liver Physiol 2006;290:G719–28. http://dx.doi.org/10.1152/ajpgi.00481.2005.

[31] Monico CG, Weinstein A, Jiang Z, Rohlinger AL, Cogal AG, Bjornson BB, et al. Phenotypic and functional analysis of human SLC26A6 variants in patients with familial hyperoxaluria and Calcium oxalate nephrolithiasis. Am J Kidney Dis Off J Natl Kidney Found 2008;52:1096–103. http://dx.doi.org/10.1053/j.ajkd.2008.07.041.

[32] Corbetta S, Eller-Vainicher C, Frigerio M, Valaperta R, Costa E, Vicentini L, et al. Analysis of the 206M polymorphic variant of the SLC26A6 gene encoding a Cl-oxalate transporter in patients with primary hyperparathyroidism. Eur J Endocrinol Eur Fed Endocr Soc 2009;160:283–8. http://dx.doi.org/10.1530/EJE-08-0623.

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08/06/2015

Péter Hegyi, MD, PhD
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HUNGARY

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Dear Dr. Hegyi,

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A Common CCK-B Receptor Intronic Variant in Pancreatic Adenocarcinoma in a Hungarian Cohort

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Objectives: Variant c.811+32C>A in intron 4 of the cholecystokinin-B receptor gene (*CCKBR*) was reported to correlate with higher pancreatic cancer risk and poorer survival. The variant was suggested to induce retention of intron 4, resulting in a new splice form 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.

Methods: We genotyped variant c.811+32C>A in 122 pancreatic adenocarcinoma case patients and 106 control subjects by sequencing and examined its association with cancer risk and patient survival. We tested the functional effect of variant c.811+32C>A on pre–messenger RNA splicing in human embryonic kidney 293T and Capan-1 cells transfected with *CCKBR* minigenes.

Results: The allele frequency of the variant was similar between patients and control subjects (18.4% and 17.9%, 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: These data indicate that variant c.811+32C>A in *CCKBR* does not have a significant impact on pancreatic cancer risk or survival in a Hungarian cohort.

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Key Words: pancreatic adenocarcinoma, CCK-B receptor, alternative splicing, survival, genetic risk factor

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Pancreatic ductal adenocarcinoma (PDAC) has the highest mortality rate of all malignancies. There is no effective screening available and advanced disease is commonly present at initial diagnosis.[1] Established risk factors are cigarette smoking, chronic pancreatitis, diabetes mellitus, and increased body mass.[2] Inherited genetic factors also play an important role in familiar and sporadic occurrences of pancreatic cancer. Several highly penetrant susceptibility genes have been identified, including *BRCA1*, *BRCA2*, *p16/CDKN2A*, *STK11/LKB*, *TP53*, *APC*, *PRSS1*, *SPINK1*, *PALLD*, and *PALB2*, which are mainly associated with familial cancer syndromes and familial pancreatic cancer.[3,4] In sporadic case patients, more common genetic variants are implicated, which represent a minor risk for the disease, such as variants in the ABO blood group gene.[5,6] To date, 5 genome-wide association studies have described multiple susceptibility loci associated with the risk of pancreatic cancer.[5–9] Smith et al[10] reported a common single nucleotide polymorphism in the cholecystokinin-B receptor gene (*CCKBR*) as a risk factor for PDAC, which has not been observed in prior genome-wide association studies. The authors showed in a small cohort (51 case patients and 39 control subjects) that variant c.811+32C>A (rs1800843) located in intron 4 of *CCKBR* increased PDAC risk and was also associated with poorer survival. In a more recent follow-up study, Smith et al[11] replicated their results in a larger North American multicenter cohort (931 case patients and 59 control subjects) and confirmed both increased PDAC risk (odds ratio [OR], 2.28; CC vs. AC plus AA genotypes) and shorter survival (hazard ratio, 1.56) associated with variant c.811+32C>A. The gastrin/CCK-B receptor is a member of the G protein–coupled receptor superfamily, physiologically expressed in the human pancreas.[12] 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 the CCK-B receptor plays a significant role in carcinogenesis and tumor progression.[13,14] An alternatively spliced messenger RNA (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 tumors,[15] including pancreatic cancer.[16] The resulting CCK-BRi4sv receptor protein exhibits constitutive (agonist independent) activation of cell proliferation pathways.[17] Using immunohistochemistry, Smith et al 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. The authors speculated that binding of the splicing factor SRp55 might be reduced by the intronic variant resulting in enhanced retention of intron 4.[12,13]

TABLE 1. Characteristics of Patients and Control Subjects

	PDAC Patients	Control Subjects
n	122	106
Sex		
Female	59	61
Male	63	45
Age at diagnosis/recruitment, y		
Mean (SD)	65.6 (10.4)	51.5 (18.8)
Range	31–89	18–89
Survival days		
Mean (SD)	336 (251.8)	
Median (25%-75%)	260 (118–465)	

There are few known risk factors in pancreatic adenocarcinoma, and a better understanding of the molecular pathogenesis is urgently needed. Therefore, we aimed to reevaluate the role of variant c.811+32C>A as a novel genetic prognostic marker. In this study, we had 3 objectives: (1) to replicate the association between *CCKBR* 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.

METHODS

Study Population

The study protocol has been approved by the Regional and Institutional Committee of Science and Research Ethics. All participants gave written informed consent for genetic analysis. A total of 122 case patients with a confirmed diagnosis of PDAC were recruited from the Hungarian National Pancreas Registry. For each patient, information about sex, age at diagnosis, method of diagnosis, and date of death or date of last follow-up was collected. Two patients had synchronous or metachronous cancer suggestive of inherited cancer syndromes. Other case patients were sporadic; no patients fulfilled the criteria for familial pancreatic cancer (2 or more first-degree relatives with pancreatic cancer). A total of 106 control subjects were recruited from adult volunteers who considered themselves generally healthy and from inpatients who had no history of pancreatic diseases. Characteristics of case patients and control subjects are described in Tables 1 and 2.

DNA Extraction and Genotyping

Genomic DNA was isolated from 300 μL of EDTA blood using a QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany). Primers were designed according to the genomic sequence of *CCKBR* on chromosome 11 (GenBank NC_000011.10) (see primer sequences in Table 3). Polymerase chain reaction (PCR) was performed in a total volume of 30 μL, which contained 0.5 U HotStarTaq DNA Polymerase (Qiagen), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each primer, and 10-50 ng of genomic DNA. Amplification was performed under the following cycle conditions: 95°C for 15 minutes to activate the enzyme, followed by 40 cycles of 30-second denaturation at 94°C, 30-second annealing at 58°C, and 1-minute extension at 72°C, with a final extension of 5 minutes. Before sequencing, PCR products were purified with a QIAquick PCR Purification Kit (Qiagen). Nucleotide sequence analysis was carried out in a commercial laboratory (Delta Bio 2000 Ltd, Szeged, Hungary) using a 3500 Genetic Analyser (Applied Biosystems) automatic dye-terminator sequencing machine. The reverse PCR primer was used as sequencing primer. Chromatograms were analyzed with ChromasPro software (Technelysium, South Brisbane, Australia).

TABLE 2. Tumor Stage and Survival of Patients

	No. Patients	Survival, Median (SD), Days
Localized	9	480 (312)
Locally advanced	78	321 (267)
Metastasized	29	222 (204)
Unknown	5	

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 (Fig. 1). *CCKBR* coding DNA (GenBank NM_176875.3) was custom synthesized (GenScript) and cloned into the pcDNA3.1(−) plasmid using *Xho*I and *Eco*RI 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 *Bsr*GI and *Bam*HI restriction sites. Full sequences of *CCKBR* minigenes are provided in the Supplementary Material, http://links.lww.com/MPA/A453.

Construction of Lentiviral Vectors

The pWPI lentivirus vector plasmid and the packaging plasmids (psPAX2 and pMD2.G) were obtained from Didier Trono's laboratory (http://tronolab.epfl.ch/; Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) through Addgene (Addgene plasmids 12254, 12260, and 12259). First, *CCKBR* minigene templates were PCR amplified with Phusion Flash High-Fidelity PCR Master Mix (Thermo Scientific) using the following primers: 5′-GCTTAATTAACCATGGAGCTGCTAAAGCTGAACC-3′ containing a *Pac*I restriction site and 5′ phosphorylated 5′-CTCAGCCAGGGCCCAGTGTG-3′. *CCKBR* minigene inserts were then subcloned into the pWPI plasmid between *Pme*I and *Pac*I restriction sites. The lentivirus production in human embryonic kidney (HEK) 293T packaging cells was performed as described previously.[18] Briefly, 293T cells were cotransfected with the pWPI expression plasmids, the packaging plasmid psPAX2, and the envelope vector pMD2.G. Transfection medium was changed after 16 hours, and the lentivirus-containing medium

TABLE 3. Oligonucleotide Primers Used in This Study

Primers Used for Genotyping		
	Forward	5′-CTGTGTTGCCTTCAGGTCCG-3′
	Reverse	5′-ATCACCAGCAACATTCGCAC-3′
Primers Used for RT-PCR		
CCKBR-total	Forward	5′-TCTCCTCAACAGCAGCAGTG-3′
	Reverse	5′-CCCAGGACCACGATGATGAG-3′
CCKB-Ri4sv	Forward	5′-AATGGAGTTGAGCTGGGAGC-3′
	Reverse	5′-TGGGCGGTCAGAGAAAAAGG-3′
GAPDH	Forward	5′-CACCATCTTCCAGGAGCGAG-3′
	Reverse	5′- GACTCCACGACGTACTCAGC-3′

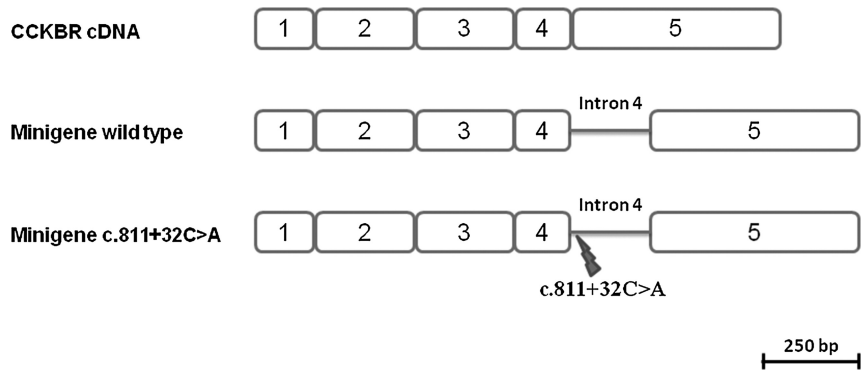

FIGURE 1. Minigene constructs used to analyze the effect of the c.811+32C>A variant on pre-mRNA splicing. Intron 4 was placed in the appropriate context of the *CCKBR* cDNA. Numbers indicate exons.

was subsequently harvested after 48 hours and frozen at −80°C. Viral preparations were titrated on HEK 293T cells.

Cell Culture, Transfection, and Viral Transduction

Human embryonic kidney 293T cells were cultured in 6-well plates in Dulbecco Modified Eagle Medium (Sigma, Budapest, Hungary) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Transfections of HEK 293T cells were performed at 70%-80% confluence using 2 μg of plasmid DNA and 10 μL of Lipofectamine 2000 (Life Technologies, Carlsbad, Calif) in 2 mL of Opti-MEM Reduced Serum Medium (Life Technologies). After 4 hours of incubation, cells were washed and the transfection medium was replaced with 2 mL of Dulbecco Modified Eagle Medium. Cells were harvested 24 hours after this medium change. Capan-1 pancreatic adenocarcinoma cells were maintained in RPMI-1640 Medium (Sigma) supplemented with 15% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin at 37°C. To establish stable cell lines, a total number of 10^5 cells were plated in 6-well plates and transduced with viral supernatant at multiplicities of infection of 4. Expression analysis was performed at the first, second, and third passage.

RNA Extraction and Reverse Transcription

Total RNA was isolated from transfected cells using an RNeasy Mini Kit (Qiagen). To avoid plasmid and genomic DNA contamination, an additional on-column DNase digestion step was applied with RNase-Free DNAse (Qiagen). Two micrograms of RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, Calif) in the presence of RNase inhibitor RNasin Plus (Promega, Fitchburg, Wis).

Quantification of *CCKBR* Expression and Splicing

Real-time PCR reactions were performed with Maxima SYBR Green/ROX qPCR Master Mix (2×) (Fermentas) on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) platform with the following conditions: 10 minutes initial denaturation at 95°C, followed by 40 two-step cycles: 15 seconds at 95°C and 1 minute at 60°C. Primer sequences are given in Table 3. Threshold cycle (CT) values were determined using the 7000 Sequence Detection System Software V.1.2.3. Relative expression was calculated using the comparative CT method (ΔΔCT method). Expression level of *CCKBR* was first normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) internal control gene (ΔCT) and then to expression levels measured in cells transfected with empty vector (ΔΔCT). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$. Relative expression of splice variants was studied by using 2 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 percentage of total (spliced plus unspliced) *CCKBR* expression. All reactions were performed in duplicate.

Statistical Analysis

Quantitative variables were described as mean ± SE. Observed genotype frequencies in the study population were compared with the expected Hardy-Weinberg equilibrium. To test the association between pancreatic cancer and genotype/allele frequencies, we used 2-tailed Fisher exact test. Additional ORs with 95% confidence interval were estimated. Overall survival was defined as the time interval between diagnosis and death (uncensored observation) or the last date when the patient was still known to be alive (censored observation). Survival curves were calculated for overall survival of patients according to Kaplan-Meier. 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. Median survival time was calculated using data from all patients; median follow-up time was computed with censored observations. All the analyses were performed with

TABLE 4. Genotype and Allele Frequencies of Variant c.811 +32C>A in PDAC Patients and Control Subjects

	PDAC Patients	Control Subjects	Genotypic OR (95% CI)	*P*
CC	82/122	71/106	Reference	—
AC	35/122	32/106	0.947 (0.5328–1.683)	0.884
AA	5/122	3/106	1.443 (0.339–6.255)	0.7271
AC + AA	40/122	35/106	0.9895 (0.5686–1.722)	1
			Allelic OR (95% CI)	
Minor allele frequency	18.4%	17.9%	1.01 (0.58–1.76)	1

CI indicates confidence interval.

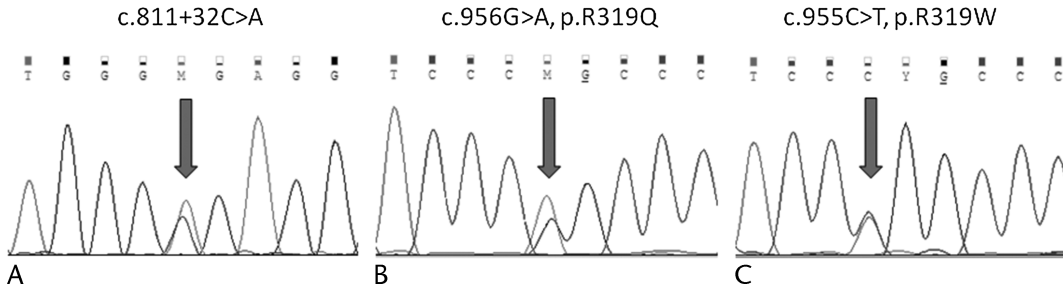

FIGURE 2. Sequence electropherograms of *CCKBR* gene variants found in our cohort.

GraphPad Prism (San Diego, Calif). For sample size calculation, we used Quanto v.1.2.4.[19]

RESULTS

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 case patients (allele frequency, 18.4%) and in 32 heterozygous and 3 homozygous control subjects (allele frequency, 17.9%). Similar to allele frequencies, genotype frequencies did not show a statistically significant difference between case patients and control subjects either (Table 4). Genotype frequencies in case patients and control subjects were found to conform to the Hardy-Weinberg equilibrium. Additionally, we identified 2 variants in exon 5: c.955C>T (p.R319W, rs113168010) in 1 control subject and c.956G>A (p.R319Q, rs1805001) in a single patient (Fig. 2).

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 (Fig. 3). Median follow-up time was 334 days; 12.3% of the observations were censored. Median survival of case patients 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 2 different cell lines. Human embryonic kidney 293T cells were transfected with expression plasmids carrying *CCKBR* minigenes with or without the intron 4 variant and examined mRNA expression. *CCKBR* expression in transfected HEK 293T cells was about 6 orders of magnitude higher than endogenously expressed levels (Fig. 4A). Interestingly, *CCKBR* mRNA was expressed at 1.5-fold higher levels when cells were transfected with intron-containing minigenes compared with cells transfected with the intronless *CCKBR* cDNA construct. This phenomenon is in agreement with published observations that the presence of introns can enhance gene expression.[20,21] For absolute quantification of different splice forms, we generated calibration curves using minigene plasmids as template. We found that expression of the *CCK-BRi4sv* intron 4–retaining splice variant corresponded to approximately 10% of total *CCKBR* mRNA and was not different between cells transfected with minigenes with or without the c.811+32C>A variant (Fig. 4C). Because 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 (Figs. 4B, D).

DISCUSSION

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.[22,23] 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.[17] 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.[24] Alternatively, Smith et al[10,14] 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.[12,13] 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 or with shorter survival in PDAC. Although we had more than 85% statistical power to replicate the previously described OR of 2.28, we detected no

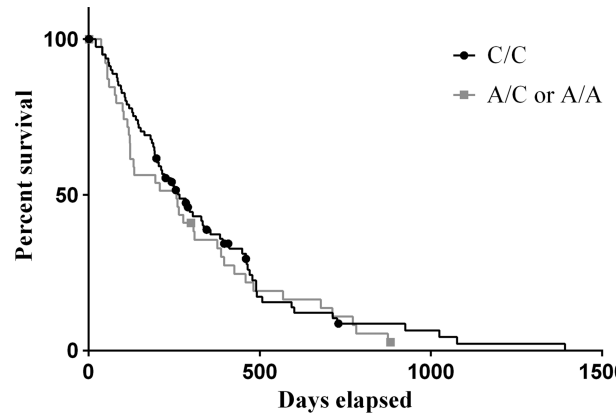

FIGURE 3. Kaplan-Meier survival curves according to genotype. Censored case patients are shown as dots and squares.

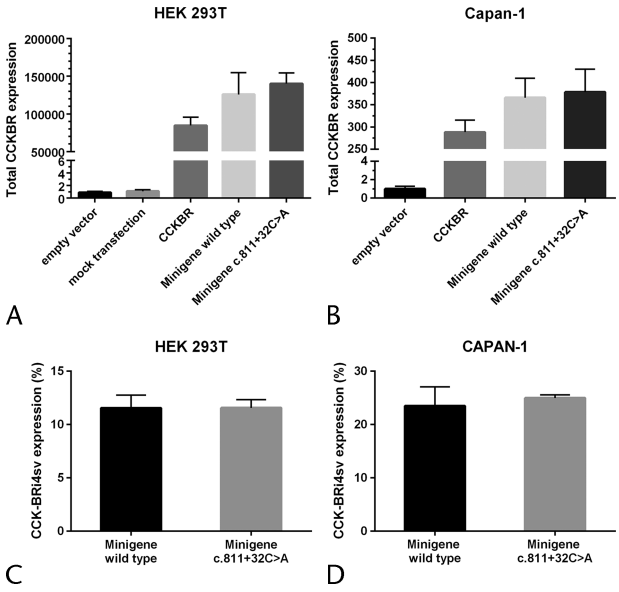

FIGURE 4. Functional analysis of the effect of variant c.811+32C>A on pre-mRNA splicing. Expression of *CCKBR* mRNA in transfected HEK 293T cells (A) and transduced Capan-1 cells (B) with the indicated constructs. Expression of the intron-retaining splice variant relative to the total amount of *CCKBR* mRNA in HEK 293T cells (C) and Capan-1 cells (D).

enrichment of the variant in our PDAC cohort. The reasons for the discrepancy between our results and those of Smith et al[10,14] are not readily apparent but may be related to ethnic and geographic variability of the frequency of the c.811+32C>A variant and the admixed nature of the US cohort. Association studies in ethnically admixed populations are potentially vulnerable to spurious association because of the ethnic variability of the single nucleotide polymorphism frequency studied. Indeed, data retrieved from the 1000 Genome Project database (www.1000genomes.org) show that the allele frequency of variant c.811+32C>A is 18.4% in subjects of European origin, whereas it is 2% in subjects of Asian descent and 23% in subjects of African descent. We also note that the control group in the study by Smith and Solomon[14] was unusually small (59 subjects), which might result in the incorrect determination of control genotype frequencies. Indeed, the reported minor allele frequency (11.8%) for this control cohort is appreciably smaller than the incidence found in our control subjects (17.9%), which compares well with the 1000 Genomes data.

In conclusion, data presented here argue that the 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.

REFERENCES

1. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362:1605–1617.
2. Lowenfels AB, Maisonneuve P. Epidemiology and risk factors for pancreatic cancer. *Best Pract Res Clin Gastroenterol*. 2006;20:197–209.
3. Marcus DM. The ABO and Lewis blood-group system. Immunochemistry, genetics and relation to human disease. *N Engl J Med*. 1969;280:994–1006.
4. Wolpin BM, Chan AT, Hartge P, et al. ABO blood group and the risk of pancreatic cancer. *J Natl Cancer Inst*. 2009;101:424–431.
5. Amundadottir L, Kraft P, Stolzenberg-Solomon RZ, et al. Genome-wide association study identifies variants in the ABO locus associated with susceptibility to pancreatic cancer. *Nat Genet*. 2009;41:986–990.
6. Low SK, Kuchiba A, Zembutsu H, et al. Genome-wide association study of pancreatic cancer in Japanese population. *PLoS One*. 2010;5:e11824.
7. Wu C, Miao X, Huang L, et al. Genome-wide association study identifies five loci associated with susceptibility to pancreatic cancer in Chinese populations. *Nat Genet*. 2011;44:62–66.
8. Petersen GM, Amundadottir L, Fuchs CS, et al. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet*. 2010;42:224–228.
9. Wolpin BM, Rizzato C, Kraft P, et al. Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat Genet*. 2014;46:994–1000.
10. Smith JP, Harms JF, Matters GL, et al. A single nucleotide polymorphism of the cholecystokinin-B receptor predicts risk for pancreatic cancer. *Cancer Biol Ther*. 2012;13:164–174.
11. Smith JP, Whitcomb DC, Matters GL, et al. Distribution of cholecystokinin-B receptor genotype between patients with pancreatic cancer and controls and its impact on survival. *Pancreas*. 2015;44: 236–242.
12. Rozengurt E, Walsh JH. Gastrin, CCK, signaling, and cancer. *Annu Rev Physiol*. 2001;63:49–76.
13. Aly A, Shulkes A, Baldwin GS. Gastrins, cholecystokinins and gastrointestinal cancer. *Biochim Biophys Acta*. 2004;1704:1–10.
14. Smith JP, Solomon TE. Cholecystokinin and pancreatic cancer: the chicken or the egg? *Am J Physiol Gastrointest Liver Physiol*. 2014;306:G91–G101.
15. Körner M, Waser B, Reubi JC, et al. CCK(2) receptor splice variant with intron 4 retention in human gastrointestinal and lung tumours. *J Cell Mol Med*. 2010;14:933–943.
16. Smith JP, Verderame MF, McLaughlin P, et al. Characterization of the CCK-C (cancer) receptor in human pancreatic cancer. *Int J Mol Med*. 2002; 10:689–694.
17. Hellmich MR, Rui XL, Hellmich HL, et al. Human colorectal cancers express a constitutively active cholecystokinin-B/gastrin receptor that stimulates cell growth. *J Biol Chem*. 2000;275:32122–32128.
18. Dawson K, Wakili R, Ördög B, et al. MicroRNA29: a mechanistic contributor and potential biomarker in atrial fibrillation. *Circulation*. 2013; 127:1466–1475, 1475e1–28.
19. Gauderman WJ, Morrison JM. QUANTO 1.1: a computer program for power and sample size calculations for genetic-epidemiology studies. http://hydra.usc.edu/gxe/ Accessed May 4, 2009.
20. Wiegand HL, Lu S, Cullen BR. Exon junction complexes mediate the enhancing effect of splicing on mRNA expression. *Proc Natl Acad Sci U S A*. 2003;100:11327–11332.
21. Kereszturi E, Király O, Sahin-Tóth M. Minigene analysis of intronic variants in common SPINK1 haplotypes associated with chronic pancreatitis. *Gut*. 2009;58:545–549.
22. Willard MD, Lajiness ME, Wulur IH, et al. Somatic mutations in CCK2R alter receptor activity that promote oncogenic phenotypes. *Mol Cancer Res*. 2012;10:739–749.
23. Schmitz F, Otte JM, Stechele HU, et al. CCK-B/gastrin receptors in human colorectal cancer. *Eur J Clin Invest*. 2001;31:812–820.
24. Ding W-Q, Kuntz SM, Miller LJ. A misspliced form of the cholecystokinin-B/gastrin receptor in pancreatic carcinoma: role of reduced cellular U2AF35 and a suboptimal 3′-splicing site leading to retention of the fourth intron. *Cancer Res*. 2002;62:947–952.

AUTHOR QUERIES

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AQ1 = Both occurrences of "Smith et al. (2012, 2014)" were changed to "Smith et al.[10,14]" (the superscript 10 and 14 being the corresponding reference citations). Is this correct?

AQ2 = "Smith et al (2014) was changed to "Smith and Solomon[14]" (the superscript 14 being the corresponding reference citation). Is this correct?

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