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Loss-of-function variant in chymotrypsin like elastase 3B (*CELA3B*) is associated with non-alcoholic chronic pancreatitis

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ABSTRACT

Background: Genetic alterations in digestive enzymes have been associated with chronic pancreatitis (CP). Recently, chymotrypsin like elastase 3B (*CELA3B*) emerged as a novel risk gene. Thus, we evaluated *CELA3B* in two European cohorts with CP.
Methods: We analyzed all 8 *CELA3B* exons in 550 German non-alcoholic CP (NACP) patients and in 241 German controls by targeted DNA sequencing. In addition, we analyzed exons 6 and 7 by Sanger sequencing and the c.129+1G>A variant by melting curve analysis in 1078 further German controls. As replication cohort, we investigated up to 243 non-German European NACP patients and up to 1665 controls originating from Poland, Hungary, and Sweden. We assessed the cellular secretion and the elastase activity of recombinant CELA3B variants.
Results: In the German discovery cohort, we detected a splice-site variant in intron 2, c.129+1G>A, in 9/550 (1.64%) CP patients and in 5/1319 (0.38%) controls (P=0.007, OR=4.4, 95% CI=1.5–13.0). In the European replication cohort, this variant was also enriched in patients (9/178 [5.06%]) versus controls (13/1247 [1.04%]) (P=0.001, OR=5.1, 95% CI=2.1–12.0). We did not find the two previously reported codon 90 variants, p.R90C and p.R90L.
Conclusions: Our data indicate that *CELA3B* is a susceptibility gene for CP. In contrast to previous reports suggesting that increased CELA3B activity is associated with CP risk, the splice-site variant identified here

Abbreviations: CELA3B, Chymotrypsin like elastase family member 3B; CP, chronic pancreatitis; NACP, non-alcoholic chronic pancreatitis.

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is predicted to cause diminished CELA3B expression. How reduced CELA3B function predisposes to pancreatitis remains to be elucidated.

1. Introduction

Chronic pancreatitis (CP) is a relapsing or persistent inflammatory condition of the pancreas characterized by progressive destruction of the pancreatic parenchyma. As a consequence, in advanced stages of the disease, maldigestion and diabetes mellitus develop due to exocrine and endocrine pancreatic insufficiency [1]. Chronic alcohol abuse is an established risk factor for CP, but over the past three decades gene mutations have emerged as major determinants of pancreatitis risk, especially in early-onset disease [2].

Premature activation of trypsin due to a disturbed balance of pancreatic proteases and their counteracting proteins has been long assumed to play a central role in the pathogenesis of the disease. Consistent with this disease concept, gain-of-function mutations in the serine protease 1 (*PRSS1*, encoding cationic trypsinogen) as well as loss-of-function variants in the serine protease inhibitor Kazal type 1 (*SPINK1*) or in the trypsin degrading enzyme chymotrypsin C (*CTRC*) have been shown to associate with CP [3–5]. However, variants in other digestive enzymes such as carboxypeptidase A1 (*CPA1*), carboxyl ester lipase (*CEL*), chymotrypsinogen B1 and B2 (*CTRB1-CTRB2*), and pancreatic lipase (*PNLIP*) have also been reported in non-alcoholic CP (NACP) patients limiting the hegemony of the trypsin-centered disease model [6–9]. In addition to alterations in these genes, which are all predominantly expressed in acinar cells, variants in ductal genes such as *CFTR*, *TRPV6*, and the *CLDN2*/*MORC4* locus were found significantly enriched in patients with NACP [10–12].

Recently, a mutation in the chymotrypsin like elastase 3B (*CELA3B*) has been reported as a novel genetic risk factor for pancreatitis [13]. CELA3B is a digestive protease, which is exclusively and abundantly expressed in the exocrine pancreas [14 and references therein]. Moore and colleagues detected by whole exome sequencing a *CELA3B* missense mutation, c.268C>T (p.R90C), in a kindred of CP [13]. The arginine at codon 90 is unique to CELA3B; all other human elastases have a leucine at the corresponding position. The overexpression of p.R90C and the revertant p.R90L variants in HEK293T cells led to increased levels of secreted as well as intracellular CELA3B, while the introduction of mutations p.L90C and p.L90R into CELA3A diminished expression. Both mutants were also more rapidly activated by trypsin. In addition, genetically engineered *Cela3b* p.R89C and p.R89L knock-in mice developed more severe pancreatitis after cerulein challenge [13]. More recently, a French study examined 644 CP patients for *CELA3B* variants and found the revertant c.269G>T (p.R90L) variant, but not the p.R90C variant, in four patients, which was absent in 566 controls [15].

Inspired by these reports, we investigated the association between *CELA3B* and CP in a large European patient cohort.

2. Methods

Study population. We investigated 847 unrelated European patients with NACP. The patients originated from Germany (n=550; 299 female), Poland (n=156; 77 female), Sweden (n=36; 21 female), and Hungary (n=105; 42 female). In addition, we analyzed up to 3202 European control subjects from Germany (n=1319), Poland (n=418 healthy controls and 1162 subjects from a local exome database of various patients without CP), Sweden (n=10), and Hungary (n=293).

The diagnosis of CP was based on two or more of the following findings: presence of a typical history of recurrent pancreatitis, pancreatic calcifications, and/or pancreatic ductal irregularities revealed by endoscopic retrograde cholangiopancreatography or by magnetic resonance imaging or computed tomography of the pancreas, or pathological sonographic findings with/without decreased pancreatic function. Patients with alcohol abuse were excluded from the study.

The institutional review boards of all participating study centers approved this study. All participants gave written informed consent.

Mutation screening. We analyzed all 8 *CELA3B* exons in 550 German NACP patients and in 241 German controls by targeted DNA sequencing. In addition, we analyzed exons 6 and 7 by Sanger sequencing and the c.129+1G>A variant by melting curve analysis in 1078 further German controls. In the European replication cohort, we investigated Polish patients and controls by whole-exome sequencing (WES), and Hungarian and Swedish samples by Sanger sequencing. For exon 7 of *CELA3B*, 105 patients and 293 controls were analyzed and reported previously [16]. WES, oligonucleotide sequences, PCR and cycle sequencing conditions are described in the **Supplementary material**.

Plasmid construction and mutagenesis. Construction of the pcDNA3.1(−) expression plasmid containing human *CELA3B* with a C-terminal His tag was described previously [14]. Missense mutations were introduced by overlap extension PCR.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells (1.5×10^6 cells per transfection) were grown at 37 °C, in Dulbecco's Modified Eagle Medium (DMEM, Gibco, catalog number 10313021) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2. Transfections were performed in 6-well tissue culture plates, in 2 mL DMEM per well, using 10 μL Lipofectamine 2000 (Invitrogen, catalog number 11668019) and 4 μg expression plasmid. Cells were incubated overnight, then the transfection mixture was removed, and the cells were rinsed with phosphate-buffered saline (pH 7.4), and covered with 1.5 mL Opti-MEM reduced serum medium (Gibco, catalog number 11058021). Conditioned medium was harvested 48 h later. CELA3B mutants were analyzed in 3–4 independent transfections.

Measurement of elastase activity. Assays were performed in duplicate. Conditioned medium (100 μL) was supplemented with 10 μL 1 M Tris-HCl (pH 8.0, 0.1 M final concentration), 1 μL 0.1 M $CaCl_2$ (1 mM final concentration), and 1 μL 5% Tween 20 (0.05% final concentration). The CELA3B precursor (proelastase) was activated with 100 nM human cationic trypsin (final concentration) for 30 min at 37 °C. Aliquots (40 μL) of activated CELA3B was then mixed with 155 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20), and elastase activity was measured by adding 5 μL of 6 mM Suc-Ala-Ala-Pro-Ala-*p*-nitroanilide (Bachem, Torrance, CA, catalog number L-1775.0250) substrate and monitoring the reaction at 405 nm for 5 min. The rate of substrate cleavage was expressed in mOD/min units. Variants p.V232L, p.A241G, and p.R247P were analyzed previously [16].

Western blotting. Conditioned medium (10 μL) was supplemented with 10 μL Laemmli sample buffer containing 100 mM dithiothreitol, heat-denatured at 95 °C for 5 min, and electrophoresed on 15% SDS-polyacrylamide gels. The proteins were then transferred to a PVDF membrane and CELA3B was detected with an anti-His tag antibody conjugated to horseradish peroxidase (penta-His HRP conjugate kit, catalog number 34460, Qiagen, Germantown, MD). The membrane was first blocked with the manufacturer's Blocking Reagent (0.5% in 1x blocking buffer with 0.1% Tween 20), followed by addition of the antibody at a dilution of 1:2000 for 1 h. HRP activity was detected with the SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific, catalog number 34580).

Statistical analysis. Differences in the frequency of variants between cases and controls were determined with two-tailed Fisher's exact test. A *p*-value of less than 0.05 was considered significant. The statistical analyses were conducted using the SPSS software (Chicago, IL; v 22) and Prism v 4.03 (GraphPad).

3. Results

Genotyping. In the German cohort, we found 1 splice-site variant and 13 non-synonymous variants, 9 of which were rare variants with an allele frequency <1% (Table 1). In the European replication cohort, we found 2 splice-site variants and 14 missense variants, including 10 rare variants.

The splice-site alteration c.129+1G>A, located in intron 2, was significantly more common in German patients compared to controls: 9/550 (1.64%) patients *vs.* 5/1319 (0.38%) controls (P=0.007, OR=4.4, 95% CI=1.5–13.1) (Table 1). The variant was also significantly associated with CP in the European replication cohort: 9/178 (5.06%) patients *vs.* 13/1247 (1.04%) controls (P=0.001, OR=5.1, 95% CI=2.1–12.0). A second splice-site variant, c.643-2A>G, was found in intron 6 of a CP patient in the European replication cohort only.

Considering missense variants, two were significantly enriched in German patients: p.R210H in exon 6 (30/550 [5.45%] patients *vs.* 34/1319 [2.58%] controls; P=0.003) and p.A241G in exon 7 (35/550 [6.36%] patients *vs.* 52/1319 [3.94%] controls; P=0.03). The two variants were found in strong linkage disequilibrium. Among the p.R210H heterozygotes, only one control subject did not have p.A241G. In contrast, p.A241G was also found without p.R210H in 5 patients and 19 controls. When we calculated the *p*-value for carriers of p.A241G without p.R210H, it became insignificant (P=0.5). The p.A241G variant was even slightly more frequent in controls: 5/550 (0.91%) patients *vs.* 19/1319 (1.44%) controls. Therefore, the significant association of p.A241G with CP is entirely due to the effect of the p.R210H_p.A241G compound heterozygotes *in cis*. Noteworthy, neither p.R210H nor p.A241G was significantly enriched in the European replication cohort (P=0.074 and 0.18, respectively) (Table 2). However, the strong linkage between these variants was replicated in this cohort too. Compound heterozygosity for variant p.A241G was confirmed in 43 of 56 carriers of p.R210H.

Variant p.I209M was slightly more frequent in control subjects in the German discovery cohort (1/550 (0.18%) *vs.* 5/1319 (0.38%), however, it was significantly enriched in CP patients of the European replication cohort (3/232 (1.29%) *vs.* 2/1665 (0.12%), P=0.015). When data from the two cohorts were combined, no significant association was calculated (P=0.26). Noteworthy, in the French study, p.I209M was not detected in 644 patients, but was present in 1/566 controls [15]. Finally, none of the other missense *CELA3B* variants showed a significant enrichment in patients or controls in either study population.

Functional analysis of CELA3B missense variants. To assess the functional consequences of *CELA3B* variants, we transiently transfected HEK293T cells and assayed elastase activity in the conditioned medium after activation with trypsin (Tables 1 and 2). This activity value represents a composite measure reflecting the secreted levels and the catalytic activity of CELA3B variants in the conditioned medium. Relative to wild-type CELA3B, we observed markedly elevated activity with variants p.W79R (172.5%) and p.R210H (201.1%), and slightly elevated activity with variants p.V203M (134.8%) and p.V232L (137.2%). Low levels of activity (<20%) were found with variants p.G163D, p.G218A, and p.T251M. All other variants exhibited normal or slightly reduced elastase activity (range 47.3%–104.3% of wild-type), including variant p.A241G, which was 47.3% as active as wild-type CELA3B. The strong linkage disequilibrium of variants p.R210H with p.A241G indicates that these are located on the same allele. Therefore, we also tested the double mutant and found that the activity fell in between those of the single mutants and was slightly higher (129.5%) than wild-type.

To examine whether the gain and loss of CELA3B function detected with a handful of mutants is related to altered expression and/or secretion, we measured the levels of secreted CELA3B in the conditioned medium by Western blots (Supplementary Fig. 2). All

Table 1
Non-synonymous and splice-site variants in German patients with non-alcoholic CP and controls.

Exon/Intron	Nucleotide change	Amino acid change	*rs number*	CP (n=550) (%)	Controls n (%)	*p*-value	OR	95% CI	Activity (% wild-type)	Activity (SD)
2	c.129+1G>A	–	*rs144994230*	9 (1.64)	5/1319 (0.38)	0.007	4.4	1.5–13.1	–	–
4	c.235T>C	p.W79R	*rs7528405*	17 (3.09)	9/241 (3.73)	0.67			172.5	14.8
5	c.401A>T	p.Q134L	*rs4272592*	1 (0.18)	0/241 (0)	1.0			98.2	13.5
5	c.415G>A	p.V139I	*rs141568613*	4 (0.73)	0/241 (0)	0.32			91	20.1
5	c.488G>A	p.G163D	*rs1158940493*	1 (0.18)	0/241 (0)	1.0			8.2	5.8
6	c.607G>A	p.V203M	*rs202009504*	1 (0.18)	0/1319 (0)	0.29			134.8	10.6
6	c.625A>G	p.I209V	*rs114365157*	43[&] (7.82)	114/1319[&] (8.64)	0.58			88	16.2
6	c.627C>G	p.I209M	*rs77941170*	1 (0.18)	5/1319 (0.38)	0.68			92.9	18.4
6	c.629G>A	p.R210H	*rs112944567*	30 (5.45)	34/1319 (2.58)	0.003	2.2	1.3–3.6	201.1	35.9
7	c.653G>C	p.G218A	*rs767121139*	1 (0.18)	0/1319 (0)	0.29			9.5	2.1
7	c.694G>C	p.V232L	*rs142871632*	2 (0.36)	5/1319 (0.38)	0.96			137.2[a]	12.7
7	c.722C>G	p.A241G	*rs114895362*	35 (6.36)	52/1319* (3.94)	0.03	1.7	1.1–2.6	47.3[a]	5.9
7	c.752C>T	p.T251M	*rs188438439*	0 (0)	1/1319 (0.08)	1.0			18.6	1.7
8	c.799A>G	p.I267V	-	3 (0.55)	1/241 (0.41)	1.0			104.3	18.9
		p.R210H_p.A241G		30 (5.45)	33/1319 (2.5)	0.002	2.3	1.4–3.7	129.5	13.8
		p.A241G (w/o p.R210H)		5 (0.91)	19/1319 (1.44)	0.50			47.3	5.9

P-values were determined by two-tailed Fisher's exact test.
[&] 1 patient and 1 control was homozygous for p.I209V; * 2 controls were homozygous for p.A241G.
[a] Activity reported previously [16].

Table 2
Non-synonymous and splice-site variants in European patients with non-alcoholic CP and controls.

Exon/Intron	Nucleotide change	Amino acid change	*rs number*	CP n (%)	Controls n (%)	*p*-value	OR	95% CI	Activity (% wild-type)	Activity (SD)
2	c.129+1G>A	–	*rs144994230*	9/178 (5.06)	13/1247 (1.04)	0.001	5.1	2.1–12.0	–	–
4	c.235T>C	p.W79R	*rs7528405*	3/76 (3.95)	1/103 (0.97)	1.0			172.5	14.8
5	c.391C>T	p.R131C	*rs149805485*	1/178 (0.56)	0/1247 (0)	0.313			51.2	15.1
5	c.392G>A	p.R131H	*rs555621832*	1/178 (0.56)	0/1247 (0)	0.313			65.9	9.1
5	c.401A>T	p.Q134L	*rs4272592*	1/220 (0.45)	0/1247 (0)	0.15			98.2	13.5
5	c.415G>A	p.V139I	*rs141568613*	0/178 (0)	1/1247 (0.08)	1.0			91	20.1
6	c.568A>G	p.R190G	-	1/232 (0.43)	0/1665 (0)	0.12			79.3	8
6	c.625A>G	p.I209V	*rs114365157*	26/232 (11.21)	183/1665 (11.11)	1.0			88	16.2
6	c.627C>G	p.I209M	*rs77941170*	3/232 (1.29)	2/ 1665 (0.12)	0.015	10.9	1.8–65.5	92.9	18.4
6	c.629G>A	p.R210H	*rs112944567*	10/232 (4.31)	38/1665 (2.28)	0.074			201.1	35.9
6	c.634G>A	p.G212S	*rs200468289*	1/232 (0.43)	1/ 1665 (0.06)	0.23			64.9	15
6	c.643-2A>G	–	*rs770659503*	1/232 (0.43)	0/1665 (0)	0.12			–	–
7	c.694G>C	p.V232L	*rs142871632*	2/243 (0.82)	12/1447 (0.83)	1.0			137.2[a]	12.7
7	c.722C>G	p.A241G	*rs114895362*	12/243 (4.94)	46/1447 (3.18)	0.18			47.3[a]	5.9
7	c.740G>C	p.R247P	-	0/243 (0)	1/1447 (0.07)	1.0			85.1[a]	5.7
8	c.799A>G	p.I267V	-	1/178 (0.56)	0/1247 (0)	0.13			104.3	18.9

P-values were determined by two-tailed Fisher's exact test.
[a] Activity reported previously [16].

loss-of-function variants (p.G163D, p.G218A, and p.T251M) showed normal or elevated elastase levels, indicating that these variants have catalytic defects. Considering variants with high elastase activity, p.W79R was normally secreted, indicating that this variant has higher catalytic activity than wild-type CELA3B. In contrast, secreted levels of p.R210H were clearly increased, which explains the high activity measured in the conditioned medium of this variant. When combined with the p.A241G variant, the double mutant showed only slightly higher secreted levels than wild-type CELA3B, which was consistent with its enzymatic activity in the conditioned medium.

Distribution of gain-of-function and loss-of-function *CELA3B* variants in patients versus controls. As described above, gain-of-function variant p.W79R showed no association with CP in either study cohort while variant p.R210H was associated with CP in the German discovery cohort but not in the European replication cohort. Considering the two variants with slightly elevated elastase activity, p.V203M was found in one CP patient only while variant p.V232L was equally distributed in patients and controls in both study populations. Loss-of-function *CELA3B* variants were rare and found in CP patients (1x p.G163D, 1x p.G218A) and controls (1x p.T251M) alike.

4. Discussion

Despite recent advances in elucidating the etiology of NACP, we are still searching for contributing genetic factors in a significant proportion of these patients. In our study, we investigated the association of *CELA3B* variants with NACP in two independent cohorts. Two previous studies reported gain-of-function variants in codon 90, p.R90C and p.R90L, in patients with CP [13,15]. Moore and co-workers performed whole exome sequencing in selected individuals of a kindred with suggested autosomal dominant inherited CP and detected the p.R90C *CELA3B* missense mutation in two affected subjects, which was absent in seven unaffected family members [13]. The fact that only two patients were genetically examined, although the family tree was significantly larger, makes it somewhat difficult to interpret the findings. More recently, a French study analyzed *CELA3B* in 644 CP patients and 566 control individuals by Sanger sequencing. The authors did not detect the p.R90C variant, but in 4 patients they found the p.R90L variant, which was absent in controls [15].

In contrast to the two previous reports, we did not find a codon 90 change in our investigated subjects. Importantly, we found in both cohorts a significant enrichment of a predicted loss-of-function variant in intron 2, c.129+1G>A. The G at position 1 of the splice donor site is highly conserved in eukaryotes. According to the Combined Annotation Dependent Depletion (CADD) web page (https://cadd.gs.washington.edu/snv), the CADD score was 35 and the SpliceAI: 0.99 (donor loss) which strongly suggests loss of function [17]. The most likely effect of the variant is skipping of exon 2 and splicing of exon 1 directly to exon 3. This would result in a frame shift with early termination and likely nonsense-mediated RNA decay. A less likely scenario is that activation of cryptic splice-site(s) occurs that would result in small insertions or deletions with negative functional consequences. Interestingly, in the German cohort, in which complete data was available, 6/9 CP patients with the *CELA3B* splice site variant were *trans*-heterozygous for mutations in known risk genes, underscoring the complex heredity of this disease. One patient was *trans*-heterozygous for *PRSS1* p.A16V, two were heterozygous and one homozygous for *SPINK1* p.N34S, and three patients presented with a *CFTR* variant: p.F508del (this patient was also heterozygous for *SPINK1* p.N34S), p.D1152H, and p.S1235R. No mutations were observed in *CEL*, *CPA1*, *CTRC*, *CUZD1*, *PNLIP*, or *TRPV6*.

The functional studies by Moore and colleagues indicate a gain-of-function of the two codon 90 variants, suggesting that increased CELA3B activity predisposes to CP. This assumption would fit well with the old concept that pancreatitis results from autodigestion due to increased activity of digestive proteases within the pancreatic parenchyma. The association of the c.129+1G>A splice-site variant in our study, which would obviously lead to a decrease in CELA3B activity, is in striking contrast to this hypothesis. To test whether the detected mutations result in reduced enzyme activity, we generated recombinant proteins for all coding variants found and analyzed their catalytic properties. We found three variants (p.G163D, p.G218A, and p.T251M) with low CELA3B activity but good expression. Their catalytic defect can be explained by the functional roles of the affected residues. Gly163 (Gly142 in conventional chymotrypsin numbering) is a critical hinge residue in the so-called activation domain of chymotrypsin-like serine proteases. Mutation of this amino acid in trypsin abolishes enzyme activity [18]. Similarly, a natural mutation of this Gly residue to Glu in coagulation factor IX results in loss of catalytic activity [19]. Gly218 (Gly196 in chymotrypsin numbering) is adjacent to the active-site Ser195 (chymotrypsin numbering) residue. Natural mutations p.G217S and p.G217R in human chymotrypsin C (CTRC) that affect this Gly residue have been found in patients with chronic

pancreatitis. Functional analysis of purified mutants revealed diminished catalytic activity (see Table 4 in Ref. [20]). Thr251 (Thr226 in chymotrypsin numbering) forms part of the primary specificity pocket of CELA3B. The specificity pocket is restricted at the mouth by Val216 (chymotrypsin numbering) and partially occluded at the bottom by Thr226, which explains the characteristic elastase-like P1 preference and the exclusion of aromatic side chains. Natural mutation p.V250E in human CTRC affects this position. This variant was found in a patient with chronic pancreatitis, and functional analysis of the purified mutant revealed less than 0.1% catalytic activity relative to the wild-type enzyme (see Table 4 in Ref. [20]). Noteworthy, in the German cohort we found these catalytically defective CELA3B variants in two patients and one control subject, while p.G163D was also detected once in the French study, but in a control subject [15]. Thus, rare *CELA3B* missense variants that lead to diminished enzymatic activity are not enriched in CP patients.

One might speculate whether functions of CELA3B other than the loss of enzyme activity predispose to NACP. The CELA3B precursor can form complexes with procarboxypeptidase A1 and even more tightly with carboxypeptidase A2. The complex formation resulted in delayed procarboxypeptidase activation *in vitro* due to an increased affinity of the inhibitory activation peptide and a consequent increase in zymogen stability [21]. In contrast, CELA3A, whose amino acid sequence is 92% identical to CELA3B, does not form stable complexes due to the evolutionary replacement of alanine at codon 241 by glycine. This codon is polymorphic in both isoforms. Although we found a significant enrichment of p.A241G in the German cohort (P=0.03), we were unable to replicate this finding in the European cohort. The lack of association in our replication cohort is in line with previous results from Hungary and France [15,16]. In the French study, p.A241G was even insignificantly less frequent in patients than in controls (3.6% *vs.* 3.7%). These data indicate that CELA3B loss of function does not predispose to CP via decreased complex formation with procarboxypeptidases.

We found a significant association between p.R210H and CP in the German cohort. Again, as in the case of p.A241G, we could not replicate our finding in the non-German cohort. Also, in the French study there was no significant association between p.R210H and CP (P=0.41) [15]. Functional analyses revealed a gain-of-function effect of the p.R210H mutant: the enzymatic activity was doubled compared to the wild-type enzyme and this was due to increased protein levels in the conditioned medium. Interestingly, we found p.R210H in strong linkage disequilibrium with p.A241G in the German cohort. This finding is in agreement with the SNiPA data (LD r^2=0.95; 1000 Genomes, Phase3 v5, European population) (https://snipa.helmholtz-muenchen.de/snipa3/) [22]. In the European replication cohort, the linkage was somewhat weaker, and this was due to the lower frequency of compound heterozygotes in the Polish cohort. These subjects were analyzed by NGS technique and the lower coverage of the CELA3 locus might explain these results.

Since the p.A241G variant resulted in a reduction in activity, we generated a p.R210H_p.A241G double mutant, which only had slightly higher enzyme activity than the wild-type enzyme.

Recently, Zhong and colleagues conducted a transcriptome-wide association study (TWAS) to identify new pancreatic cancer susceptibility genes. This study showed that genetically predicted *CELA3B* expression correlates inversely with the risk of pancreatic cancer. Pathway enrichment analysis suggested that low *CELA3B* expression may be associated with an inflammatory state in the pancreas [23]. This observation might support the association we reported here between CP and variant c.129+1G>A, which is likely to result in decreased levels of CELA3B.

In summary, our observations further support that *CELA3B* represents a risk gene for CP. Contrary to previous reports, we found evidence that loss of function rather than increased CELA3B activity predisposes to CP. However, how a reduction in CELA3B contributes to the pathophysiology of NACP remains enigmatic.

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Author contributions

M.E., E.H., M.S.-T., and H.W. conceived and directed the study.

T.G., E.H., A.P., R.P., A.M.R., A.T., L.S.U., and F.Z. performed genotyping, genetic data collection and bioinformatical analysis.

P.B., S.G., P.H., D.K., F.L., H.L., J.-M.L., G.O., A.P., J.R., and H.W. recruited study subjects, collected clinical data contributed to analysis and interpretation of data.

A.D. performed functional analyses of CELA3B variants.

M.E., E.H., M.S.-T., and H.W. drafted and revised the manuscript with substantial help from J.R.

All authors approved the final manuscript and contributed critical revisions to its intellectual content.

Declaration of competing interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2022.06.258.

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